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FINAL REPORT - PROVISIONAL ALGAL
ASSAY PROCEDURES

D. F. Toerien, et al

California University
Berkeley, California

October 1971

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Final Report
PROVISIONAL ALGAL
ASSAY PROCEDURES



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FINAL REPORT
PROVISIONAL ALGAL ASSAY PROCEDURES

By

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for the

WATER QUALITY OFFICE
ENVIRONMENTAL PROTECTION AGENCY
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and
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University of California
Berkeley

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ABSTRACT

Batch and continuous flow (chemostat) assays were investigated as part of a joint industry-government sponsored, multilaboratory effort to develop a standardized algal assay procedure for nutrient level assessment. Assays were conducted with Selenastrum capricornutum as a standard assay organism.

Batch culture assays were found to have a lower level of precision than chemostat assays in the assessment of growth response as a function of nutrient concentration. The biomass parameter, maximum cell concentration, $\hat{X}$, of the batch assay generally responded to the nutrient concentration of the sample; however, the chemostat biomass parameter, steady state cell concentration, X_1 , always was found to be proportional to the nutrient concentration of the samples.

The results of spiking tests with batch assays generally were inconclusive with respect to identification of the growth rate limiting nutrient whereas the results of spiking tests with chemostats indicated clearly the growth rate limiting nutrient. It is recommended that batch type algal assays be used only for crude screening or routine monitoring purposes and that the chemostat should be used for the quantitative assessment of the algal growth supporting properties of waters as well as for the development of kinetic descriptions for nuisance algae and the rate limiting nutrients of concern.

A kinetic description of Selenastrum capricornutum indicated a low half saturation constant, K_s , (the concentration of nutrient supporting one-half the maximum growth rate) of about $5 \mu\text{g P/l}$ for phosphate phosphorus and a yield coefficient, Y , that varied as a function of growth rate. A theoretical model was proposed and evaluated which describes the varying yield coefficient (the result of "excess" uptake) as a function of the growth rate (mean cell residence time). The function was verified experimentally at a very high statistical confidence level. The significance of these findings and their application to the practical problem of eutrophication assessment is presented.

This report is submitted in accordance with the requirements of Grant No. 16010 DQB of the Water Quality Office, Environmental Protection Agency.

CONTENTS

<u>Section</u>		<u>Page</u>
I	Conclusions	1
II	Recommendations	11
III	Introduction	15
IV	Theory and Rationale	25
V	Materials and Methods	59
VI	Results and Discussion	71
VII	Comparison of Batch and Chemostat Algal Assays	161
VIII	Acknowledgments	167
IX	References	169
X	Publications and Patents	179
XI	Glossary	181
XII	Appendices	183

v
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FIGURES

		<u>PAGE</u>
1	ALGAL GROWTH MODEL FOR NATURAL AQUATIC SYSTEMS	16
2	A GRAPHICAL COMPARISON OF MICHAELIS-MENTEN, MONOD EQUATION, EXPONENTIAL EQUATION AND BLACKMAN MODEL WITH THE SAME VALUES OF MAXIMUM SPECIFIC GROWTH RATE AND HALF SATURATION CONSTANT	29
3	IDEAL BATCH GROWTH CURVE	33
4	BATCH ALGAL GROWTH IN UNRESTRICTED NUTRIENT ENVIRONMENTS	35
5	BATCH ALGAL GROWTH UNDER SPECIFIC GROWTH RATE LIMITING CONDITIONS	36
6	CONTINUOUS FLOW STIRRED TANK REACTOR SYSTEM (CFSTR)	40
7	THEORETICAL STEADY STATE CONDITIONS IN CHEMOSTAT	43
8	COMPARISON OF NUTRIENT REMOVAL VELOCITY AND NUTRIENT UTILIZATION RATE OF NO "EXCESS" AND "EXCESS" NUTRIENT UPTAKE CONDITIONS	46
9	DEFINITION SKETCH FOR A SPECIFIC NUTRIENT SUPPLY, UPTAKE AND UTILIZATION FOR GROWTH IN A CHEMOSTAT AT STEADY STATE	48
10	COMPONENT NUTRIENT CONCENTRATIONS FOR VARYING MEAN CELL AGES FOR A SPECIFIC NUTRIENT IN STEADY STATE CHEMOSTAT OPERATION WHEN "EXCESS" NUTRIENT UPTAKE OCCURS	49
11	GRAPHICAL COMPARISON OF EXPONENTIAL FUNCTION AND RECTANGULAR HYPERBOLIC FUNCTION FOR NET YIELD COEFFICIENT - CELL AGE RELATIONSHIP (SAME VALUE OF MAXIMUM CELL YIELD) Y_{MAX}	52
12	THE FRACTION OF EXCESS NUTRIENT UPTAKE AS A FUNCTION OF MEAN CELL AGE (COMPARISON OF EXPONENTIAL AND HYPERBOLIC MODELS)	54

	<u>PAGE</u>
13 THEORETICAL STEADY STATE CONDITIONS IN CHEMOSTAT WITH A VARYING YIELD COEFFICIENT (EXCESS NUTRIENT UPTAKE)	57
14 AIR TREATMENT AND DISTRIBUTION SYSTEM	66
15 BATCH ASSAY UNIT	67
16 DIAGRAM OF CHEMOSTAT ASSAY UNIT	68
17 FLOW DIAGRAM OF ROUTINE ANALYSES PERFORMED ON CHEMOSTATS	70
18 RELATIONSHIP OF <u>SELENASTRUM CAPRICORNUTUM</u> RESPONSE (SUSPENDED SOLIDS) TO CONCENTRATION OF PAAP MEDIUM	81
19 RELATIONSHIP OF <u>SELENASTRUM CAPRICORNUTUM</u> RESPONSE (CELL NUMBERS) TO CONCENTRATION OF PAAP MEDIUM	82
20 RELATIONSHIP OF <u>SELENASTRUM CAPRICORNUTUM</u> RESPONSE (TOTAL CELL VOLUME) TO CONCENTRA- TION OF PAAP MEDIUM	84
21 RELATIONSHIP BETWEEN DILUTION OF CLEAR LAKE WATER AND $\bar{x}$ BASED ON SUSPENDED SOLIDS	91
22 RELATIONSHIP BETWEEN DILUTION OF CLEAR LAKE WATER AND $\bar{x}$ BASED ON CELL NUMBERS	91
23 RELATIONSHIP BETWEEN DILUTION OF CLEAR LAKE WATER AND $\bar{x}$ BASED ON TOTAL CELL VOLUME	92
24 MAXIMUM BATCH GROWTH RATES (μ_b) IN CLEAR LAKE WATER	94
25 EFFECT OF SECONDARY EFFLUENT ADDITION ON $\bar{x}$ BASED ON SUSPENDED SOLIDS OF CLEAR LAKE WATER	99
26 EFFECT OF SECONDARY EFFLUENT ADDITION ON $\bar{x}$ BASED ON CELL NUMBERS OF CLEAR LAKE WATER	99

	<u>PAGE</u>
27 EFFECT OF SECONDARY EFFLUENT ADDITION ON $\bar{X}$ BASED ON TOTAL CELL VOLUME OF CLEAR LAKE WATER	100
28 EFFECT OF SECONDARY EFFLUENT ON THE ALGAL GROWTH RATE OF CLEAR LAKE WATER	102
29 EFFECT OF ENRICHMENT (SPIKING) ON $\bar{X}$ OF CLEAR LAKE WATER	104
30 RELATIONSHIP BETWEEN DILUTION OF SACRAMENTO RIVER WATER AND $\bar{X}$ BASED ON SUSPENDED SOLIDS	107
31 RELATIONSHIP BETWEEN DILUTION OF SACRAMENTO RIVER WATER AND $\bar{X}$ BASED ON CELL NUMBERS	107
32 RELATIONSHIP BETWEEN DILUTION OF SACRAMENTO RIVER WATER AND $\bar{X}$ BASED ON TOTAL CELL VOLUME	108
33 MAXIMUM BATCH GROWTH RATE (μ_b) IN SACRAMENTO RIVER WATER	110
34 EFFECT OF SECONDARY EFFLUENT ADDITION ON $\bar{X}$ BASED ON CELL NUMBERS OF SACRAMENTO RIVER WATER	116
35 EFFECT OF SECONDARY EFFLUENT ADDITION ON $\bar{X}$ BASED ON TOTAL CELL VOLUME OF SACRAMENTO RIVER WATER	116
36 EFFECT OF SECONDARY EFFLUENT ADDITION ON $\bar{X}$ BASED ON SUSPENDED SOLIDS OF SACRAMENTO RIVER WATER	117
37 EFFECT OF SECONDARY EFFLUENT ADDITION ON μ_b OF SACRAMENTO RIVER WATER	117
38 EFFECT OF ENRICHMENT (SPIKING) ON THE $\bar{X}$ OF SACRAMENTO RIVER WATER	119
39 GROWTH CURVES OF ENRICHMENTS (SPIKES) OF SACRAMENTO RIVER WATER	121

	<u>PAGE</u>
40 RELATIONSHIP BETWEEN CELL BIOMASS AND PHOSPHORUS UPTAKE FOR DIFFERENT RESIDENCE TIMES	126
41 OPERATION OF CHEMOSTATS 1, 2, AND 3	127
42 OPERATION OF CHEMOSTATS 4, 5, AND 6	128
43 OPERATION OF CHEMOSTATS 7, 8, AND 9	129
44 OPERATION OF CHEMOSTATS 10, 11, AND 12	130
45 RELATIONSHIP BETWEEN STEADY STATE CELL CONCENTRATION AND PHOSPHATE UPTAKE AT INFINITE HYDRAULIC RESIDENCE TIME	140
46 NET CELL YIELD COEFFICIENT AS A FUNCTION OF MEAN CELL AGE, θ_c	141
47 COMPARISON OF NUTRIENT REMOVAL VELOCITY, q , AND SPECIFIC GROWTH RATE, μ	143
48 FRACTION OF "EXCESS" PHOSPHORUS UPTAKE AS FUNCTION OF MEAN CELL AGE, θ_c	145
49 PLOT OF MICHAELIS-MENTEN (MONOD) EQUATION FOR PHOSPHORUS LIMITATION	147
50 RELATIONSHIP BETWEEN STEADY STATE CELL CONCENTRATION AND DILUTION OF SACRAMENTO RIVER WATER	153
51 SACRAMENTO RIVER CHEMOSTAT CELL CONCENTRATION, X_1 , VARIATION WITH DIFFERENT SPIKING TREATMENTS	159

TABLES

<u>No.</u>		<u>Page</u>
I	Macronutrient Composition of 100% PAAP Medium	60
II	Micronutrient Composition of 100% PAAP Medium	60
III	Accuracy and Precision of Analytical Methods and Biomass Estimates	64
IV	Removal of Algae from PAAP Medium by Pasteurization and Filtration	72
V	Removal of Indigenous Algae from a Natural Sample by Pretreatments	73
VI	Pretreatment Effect on the Chemical Composition of Samples	74
VII	Mean Algal Growth Rates Obtained After Sample Pretreatment	76
VIII	Mean Cell Production ($\bar{X}$) Values Obtained After Sample Pretreatment	77
IX	Mean Values and Precision Estimates for $\bar{X}$ Based on Different Biomass Parameters	85
X	Experimental Procedure for Identification of Growth-Limiting Nutrient	89
XI	Means and Precision of Maximum Cell Concentration Estimates in Clear Lake Water	93
XII	Means and Precision of Algal Growth Rate Estimates for Clear Lake Water	95
XIII	Means of Inorganic Nitrogen and Phosphorus Concentrations in Clear Lake Water	96
XIV	Effect of Nutrient Enrichment (Spikes) on Mean Specific Growth Rate, Batch ($\hat{\mu}_b$) of Clear Lake Water	106
XV	Algal Growth Rate Estimates, $\hat{\mu}_b$, for Sacramento River Water	111

		<u>Page</u>
XVI	Inorganic Nitrogen and Phosphorus Concentrations in Sacramento River Water	112
XVII	Effect of Nutrient Enrichment (Spikes) on Mean Specific Growth Rate, Batch (μ_b) of Sacramento River Water	120
XVIII	Conditions for Chemostat Assays	125
XIX	Precision of Chemostat Bioassay Procedure	132
XX	Summary of Chemostat Runs A and B	133
XXI	Summary of Chemostat Runs C and D	134
XXII	Summary of Chemostat Runs E and F	135
XXIII	Summary of Chemostat Runs G and H	136
XXIV	Summary of Chemostat Runs I and J	137
XXV	Kinetic Growth Models and Constants for <u>Selenastrum capricornutum</u> Under Phosphorus Limiting Conditions (Various Models and Computational Methods)	149
XXVI	The Most Probable Kinetic Growth Models and Best Estimate of Growth Constants and Coefficients for <u>Selenastrum capricornutum</u> Under Phosphorus Limited Conditions	150
XXVII	Summary of Chemostat Operation Results on Sacramento River Water	152
XXVIII	Presumed Steady State Chemostat Parameters for Sacramento River Water	155
XXIX	Chemostat Enrichment (Spiking) Procedure and Observed Data with Sacramento River Water	158

SECTION I

CONCLUSIONS

SAMPLE PRETREATMENT

1. Pasteurization and Whatman No. 41 and membrane (0.45- μ pore-size) filtration removed test algae from simulated natural waters based upon the absence of algal growth in treated samples.
2. Heat treatment, i. e., pasteurization and autoclaving, and membrane filtration removed indigenous algae from natural water sample. However, Whatman No. 41 filtration did not appear to remove the indigenous algae.
3. The chemical composition of samples apparently is not substantially changed by heat treatment; however, filtration of samples removed particulate matter from the samples which may reduce available nutrient content.
4. The reduction of nutrient content by filtration techniques (especially membrane filtration) resulted in significantly lower maximum cell concentrations ($\bar{X}$) and maximum specific growth rates, batch ($\hat{\mu}_b$) ($p = 0.95$) in the filtered samples as compared to heat treated samples.
5. The removal of particulate matter from a sample appeared to reduce the overall available nutrient concentrations of the sample, thereby causing an underestimate of the $\bar{X}$ of that sample. The release of previously bound nutrients (in particulate matter) by heat treatment of samples increased the $\hat{\mu}_b$ significantly. In the original untreated water these nutrients were not immediately available for algal growth and hence the $\hat{\mu}_b$ was overestimated.

INTERLABORATORY PRECISION TEST

6. The use of foam rubber stoppers in batch assays can lead to toxic conditions which affect algal growth. This toxicity appeared to persist even after thorough washing procedures and caused growth inhibition in the same flasks during subsequent assays.
7. To determine whether or not algal growth responses in assays were affected by foam rubber toxicity, the foam rubber stoppers require cumbersome evaluation which reduces the suitability of such stoppers for batch assays.
8. Nontoxic stoppers, or glass beakers, etc., should be used in batch assays rather than foam rubber stoppers.

9. The $\hat{X}$ in full strength (100%) PAAP medium appeared to be limited by something other than dissolved nutrients in the medium because a proportional increase of $\hat{X}$ with increase in nutrient concentration was not observed.
10. The growth limitation likely was caused by a limitation in the availability of carbon (CO_2) in the high nutrient concentration samples, as evidenced by the high pH values reached in these assays.
11. Carbon limitation apparently can occur in high nutrient content samples even when aeration is practiced. The use of specific surface area to volume ratio flasks is no guarantee that carbon limitation will not occur in high nutrient content samples. Even when aeration is used, the pH of cultures must be kept between 7.0 and 8.3 by increasing or decreasing air flow rates (or addition of CO_2) to prevent carbon from becoming limiting or the samples from having too low a pH.
12. The $\hat{X}$ responses for enriched samples (PAAP medium with initial concentrations of more than 62 $\mu\text{g P/l}$ and 1400 $\mu\text{g N/l}$) as measured in terms of suspended solids (mg/l), cell numbers ($\text{cells} \times 10^6/\text{ml}$) or total cell volume ($\mu^3 \times 10^6/\text{ml}$) were similar, indicating that all three methods were adequate parameters of biomass.
13. The adequacy of $\hat{X}$ responses for assays with low concentrations of nutrients (i. e., $\ll 62 \mu\text{g P/l}$ and $\ll 1400 \mu\text{g N/l}$) has yet to be demonstrated.
14. The fact that there was virtually no difference between the $\hat{\mu}_b$ values in the different concentrations of the PAAP medium tested (i. e., 10%, 30%, and 100%) is explained by the fact that the nutrient level of the medium was so high, at least more than 10 times the K_s (half saturation constant) value for Selenastrum capricornutum, that no differences should be expected. It does not indicate that $\hat{\mu}_b$ is a useless algal response.
15. The precision of $\hat{X}$ and $\hat{\mu}_b$ estimates in the enriched media assays were adequate, i. e., they had coefficients of variations of 15% or less; values which are acceptable for biological assay techniques.
16. The results of the interlaboratory precision test suggest that the batch bioassay method can be used with reasonably enriched samples in eutrophication analyses, at least as a screening procedure, because $\hat{X}$ and $\hat{\mu}_b$ can be estimated fairly precisely (coefficient of variation approximately 15%).

NATURAL WATER SAMPLES

17. The $\hat{X}$ response with the batch culture assay was reasonably proportional to the concentration of the water samples as illustrated

by the linear relationships between $\hat{X}$ responses and the concentration (30% or more) of either Clear Lake or the Sacramento River.

18. The linear relationships between $\hat{X}$ and concentrations of the natural waters illustrated the absence of toxicity in both waters.

19. The precision (coefficient of variation $\leq 18.5\%$) of $\hat{X}$ responses in the natural samples indicated that $\hat{X}$ can be estimated fairly precisely in natural water samples (e. g., Sacramento River) having nutrient concentrations in the order of $34 \mu\text{g P/l}$ and $133 \mu\text{g N/l}$. The precision of $\hat{\mu}_b$ responses was adequate (coefficient of variation $\leq 15\%$) except in one triplicate set of flasks.

20. Clear Lake (a eutrophic lake with $387 \mu\text{g P/l}$ and $190 \mu\text{g N/l}$) water must be diluted more than 50% before the $\hat{\mu}_b$ is appreciably reduced; however, dilution of Sacramento River water reduced the $\hat{\mu}_b$ even at one-third dilution.

21. The low initial inorganic N/P ratios of Clear Lake (0.49) and Sacramento River (3.9) indicated that nitrogen rather than phosphorus was the growth-limiting nutrient; this was borne out by the yield coefficients for phosphorus (Y_P) in the assays. However, the yield coefficients for nitrogen (Y_N) did not indicate that nitrogen limited growth in the samples, possibly because hydrolysis of nitrogenous particulate matter contributed nitrogen for cell synthesis.

22. The use of yield coefficients in batch assays for identifying the growth-limiting nutrient is limited both by the release of nutrients from organic particulate matter as well as by variations of yield coefficients in batch culture under different culture conditions.

23. The addition of secondary effluent up to 30% (V/V) increased the $\hat{X}$ responses of Clear Lake and Sacramento River linearly but $\hat{\mu}_b$ estimates showed toxic effects of such additions at the 20% and 30% levels in the case of Clear Lake. The $\hat{\mu}_b$ of Sacramento River increased with increased concentration of secondary effluent.

24. The solution of eutrophication problems depends heavily on the identification of the growth-limiting nutrients in receiving waters and wastewater-receiving water mixtures because only then can one be certain as to which nutrient, if any, must be removed or reduced in concentration. The amount of reduction needed can only be determined from the growth kinetics of algae, i. e., knowledge of the maximum specific growth rates ($\hat{\mu}$), half saturation constants (K_s), yield coefficients (Y), decay rates (k_d), and other growth parameters for the algae of concern.

25. The $\hat{\mu}_b$ measurements in wastewater-receiving water assays indicated the presence of toxicity which was not indicated by $\hat{X}$ measurements in this investigation.

26. Enrichment (spiking) techniques to identify the growth-limiting nutrient in natural waters may result in anomalous results based on $\bar{X}$ or $\hat{\mu}_b$ estimates. Because micronutrients apparently were important as growth-limiting nutrients in the waters studied, the amount of contamination in laboratory macronutrient chemicals could have caused these anomalies. Spiking procedures should be evaluated more thoroughly than was possible in this investigation.

27. The present status of the batch assay is that of a screening device, and possibly as a routine monitoring method. However, its direct application to the solution of practical problems within a rational framework is not yet apparent.

28. Further research on the batch assay is needed to establish its value for determining algal growth kinetics, and to provide a rational framework for applying the results to control the algal standing crop in the receiving waters.

CONTINUOUS CULTURE STUDIES

29. Phosphorus is the sole rate-limiting nutrient when N/P ratios are greater than 40 and when all other macronutrients and micronutrients are in excess.

30. "Excess" phosphorus uptake does occur for Selenastrum capricornutum in the continuous culture reactor (chemostat). Theoretical relationships for "excess" nutrient uptake were developed and evaluated experimentally with the following results:

a. Net cell yield (Y_n) - cell age (θ_c) relationship

$$Y_n = \frac{X_1}{S_0 - S_1} = Y_{\max} \left(1 - e^{-\theta_c / K_A} \right) = 805 \left(1 - e^{-\theta_c / 4.78} \right)$$

where $Y_{\max}$ = maximum yield coefficient, K_A = nutrient (phosphorus) utilization constant.

b. Fraction of excess nutrient (phosphorus) uptake (f_e) - cell age (θ_c) relationship

$$f_e = \frac{S_e}{S_0 - S_1} = e^{-\theta_c / K_A} = e^{-\theta_c / 4.78}$$

where S_e = amount of "excess" nutrient (phosphorus) uptake.

31. The maximum specific growth rate, $\hat{\mu}$, is related to "washout" residence time θ_w as follows:

$$\hat{\mu} = \frac{1}{\theta_w} + k_d$$

where k_d is the decay constant of the organism, time^{-1} . For Selenastrum capricornutum in continuous cultures k_d was found to be 0.001 day^{-1} , $\theta_w = 0.54 \text{ day}$, and $\hat{\mu} = 1.85 \text{ day}^{-1}$.

32. The cell continuity equation ($1/\theta_c = Yq - k_d$) can be modified to a net cell yield coefficient - cell age relationship to account for the varying yield coefficient (i. e., "excess" nutrient uptake) as follows:

$$\frac{1}{\theta_c} = Yq - k_d = Y_n \left(1 + k_d \theta_c \right) \left(\frac{S_o - S_1}{X_1 \theta_c} \right) - k_d$$

and

$$Y_n = \frac{X_1}{S_o - S_1} = Y_{\max} \left(1 - e^{-\theta_c / K_A} \right)$$

33. Under phosphorus-limited conditions, the specific growth rate - phosphorus concentration relationship can be described by the Michaelis-Menten, Monod equation

$$\mu = \hat{\mu} \left(\frac{S_1}{K_s + S_1} \right)$$

where μ = (gross) specific growth rate, time^{-1}
 $\hat{\mu}$ = maximum specific growth rate, time^{-1}
 S_1 = limiting nutrient (phosphorus) concentration, M/V
 K_s = half saturation constant, M/V.

34. A linear least square regression analysis can be successfully used for any linear equation and any linear transformation from a nonlinear equation. However, to eliminate any increase in the computational error by transformations, a direct nonlinear regression analysis (computer program CAL G2 NLIN, Appendix B) was used and it can be applied to any nonlinear equation.

35. The kinetic analysis of the chemostat results suggest the following kinetic equations and constants under phosphorus-limited conditions for Selenastrum capricornutum

Kinetic Growth Relationship	Best Estimated Value and Equations
Net yield coefficient-cell age relationship	$Y_n = \frac{X_1}{S_0 - S_1} = Y_{\max} \left(1 - e^{-\theta_c / K_A} \right)$
(Modified Cell Continuity Equation)	$Y_{\max} = 805 \frac{\text{mg cell produced}}{\text{mg P utilized}}$ $K_A = 4.78 \text{ days}$
Fractions of excess phosphorus uptake-cell age relationship	$f_e = e^{-\theta_c / K_A}$ $K_A = 4.78 \text{ days}$
Specific growth rate-rate-limiting nutrient concentration relationship	Michaelis-Menten = Monod equation $\mu = \frac{\hat{\mu} S_1}{K_s + S_1}$ $k_d = 0.001 \text{ day}^{-1}$ $\hat{\mu} = 1.85 \text{ day}^{-1}$ $K_s = 3.7 \sim 5.7 \mu\text{g/l as P}$

36. The K_s for phosphorus-limited conditions was found to be quite low ($K_s = 3.7 \sim 5.7 \mu\text{g P/l}$). This indicates that the low phosphate concentrations found in many natural waters ($< 30 \mu\text{g P/l}$) would support the growth of Selenastrum capricornutum at rates between 0.9 and $\sim 1.8 \text{ day}^{-1}$. Note that $\hat{\mu}$ was found to be 1.85 day^{-1} . The low value obtained for K_s for phosphorus and Selenastrum capricornutum indicates that many waters with high phosphorus concentrations (i. e., $\geq 50 \mu\text{g P/l}$) have phosphorus in considerable excess and are not likely limited by phosphorus for organisms with characteristics similar to Selenastrum capricornutum.

37. The kinetics of algal growth response to phosphorus-limited condition can be determined easily with continuous flow algal chemostats. Using appropriate kinetic models, it is possible to evaluate the kinetic models and estimate the kinetic constants and coefficients for algal systems (organisms and rate-limiting nutrients) and the results can be applied and interpreted directly to practical eutrophication problems.

38. The half saturation constant, K_s , along with the maximum specific growth rate, $\hat{\mu}$, for particular algal species and nutrients are the most important parameters in algal growth kinetics and their practical application. The chemostat assay is the only procedure currently known and evaluated that will yield the required kinetic information for the algal species and nutrients of concern.

CHEMOSTAT STUDIES - SACRAMENTO RIVER WATER

39. A linear relationship between the steady state cell concentration, X_1 , and the concentration of Sacramento River water illustrated the practical applicability of the chemostat assay procedure in studies of relative algal response and toxicity evaluation. For Sacramento River water, the linearity of steady state cell concentration and the dilution of the river water was:

Cell counts ($\times 556$ cells/ml) = $10.8 + 1.34\%$ Sacramento River water with a correlation coefficient of $r = 0.97$ and $n = 9$. No toxic effect on growth was observed.

40. The use of the yield coefficient in identifying the growth rate limiting nutrient was limited. However, some positive conclusions could be made from the yield coefficients with respect to certain nutrients. For example, with Sacramento River water, nitrogen was not the limiting nutrient because the nitrogen yield coefficients were markedly lower than those that have been observed for nitrogen-limited systems.

41. It appears that the most useful growth constants is the half saturation constant, K_s . It can be used not only in identifying the growth-limiting nutrient but also in the practical application of kinetic data to real problems. Based upon the values obtained for K_s for phosphorus, it can be positively concluded that phosphorus was not the growth-limiting nutrient in Sacramento River water. However, no definite conclusion can be made with respect to nitrogen based on K_s values due to the lack of a reliable description of Selenastrum capricornutum with respect to nitrogen-limited conditions.

42. A chemostat enrichment (spiking) procedure was used to identify the growth-limiting nutrient in Sacramento River water. Nitrogen, phosphorus, or iron were shown not to limit growth, whereas one of the micronutrients in a micronutrient mix appeared to limit the growth of Selenastrum capricornutum in Sacramento River water. The chemostat spiking procedure appeared to be the easiest, quickest, and most reliable of all spiking methods tested for the identification of the growth-limiting nutrient.

COMPARISON OF BATCH AND CHEMOSTAT ASSAYS

43. The biomass response of both the batch and the chemostat assays was linearly related to the concentration of the growth-limiting nutrient. The chemostat, however, had the highest precision.
44. The chemostat is more suitable than the batch assay for determining the growth rate-nutrient concentration relationships of Selenastrum capricornutum.
45. The use of yield coefficients appeared to be of no value in identifying the growth rate limiting nutrient in batch assays; however, with chemostats it appeared to have promise once the relationships between the yield coefficient with "excess" uptake and the growth rate have been established as was done for phosphorus in this study.
46. Enrichment (spiking) techniques employed in this study were of questionable value in batch assays when used to identify the growth-limiting nutrient; however, with the chemostats they appeared to identify the growth-limiting nutrient in question.
47. The K_s determinations are without value in batch assays for identifying the growth-limiting nutrient; however, in chemostats they can be extremely helpful.
48. Batch culture assays were markedly inferior to chemostats for determining the growth kinetics of algae.
49. Batch culture assays have less equipment requirements than chemostat assays and are simpler to conduct. However, chemostat assays are more versatile and yield more valuable information with higher precision than batch culture assays. This is especially true for the important kinetic relationships.
50. The operational costs and time requirements of batch culture assays and chemostat assays were not significantly different.

ROLE OF BATCH CULTURE ASSAYS IN EUTROPHICATION ANALYSES

51. Batch culture assays, being inferior to chemostat assays in most respects, appear to be best suited for screening and routine monitoring purposes.

ROLE OF CHEMOSTAT ASSAYS IN EUTROPHICATION ANALYSES

52. Chemostat assays have a multiple role in eutrophication analyses.

a. The primary role of chemostats is for determination of the kinetic characteristics (μ , K_s , Y_n , Y_{max} , k_d , K_A , and K_B) of the specific algae and nutrients of concern. In this role the chemostat assay is the only one to date that has yielded reliable and statistically significant results.

b. The second role for the chemostat is in the identification of the growth rate limiting nutrients by appropriate enrichment (spiking) procedures as outlined and evaluated in this study.

c. The third but by no means an insignificant role for the chemostat is the determination of the relative nutrient concentration in waters or wastes. It has been demonstrated adequately that at a given (fixed) residence time the steady state level of algal cells, X_1 , in the chemostat is linearly and solely related to the concentration of the rate-limiting nutrient concentration in the feed stream (sample).

SECTION II

RECOMMENDATIONS

SAMPLE PRETREATMENT

1. Filtration through Whatman No. 41 filter paper should not be used as a sample pretreatment method because its ability to remove indigenous algae is questionable.
2. The use of electronic particle counter algal counts for heat-treated, nonfiltered samples must be made with caution because of the possibility of artifact counts due to debris which contributes to the total count.
3. Heat treatment of samples, i. e., pasteurization and autoclaving (without a subsequent filtration step), is recommended for samples when the $\hat{X}$ is to be estimated. Membrane filtration (0.45- μ pore size) of the samples is recommended when $\hat{\mu}_b$ is to be estimated. Of the two heat treatments evaluated, autoclaving is recommended because of its simplicity.

INTERLABORATORY PRECISION TEST

4. The use of foam rubber stoppers in batch assays is not recommended because other nontoxic closures, such as glass beakers, can be used without extensive toxicity studies.
5. Carbon limitation can reduce the $\hat{X}$ in high-nutrient concentration samples with batch assays, hence ventilation or aeration (with or without CO₂ addition) is recommended for batch assays because the use of specific surface area to volume ratios does not insure that carbon limitation will not occur.
6. When aeration or specific surface area to volume ratios are used in batch assays, the pH must be kept between 7.0 and 8.3. With ventilation or aeration this can be accomplished by increasing or decreasing air flow rates or by addition of CO₂ to the air as required.
7. The use of suspended solids, total cell volume, or cell numbers appears to be adequate for parameters of biomass; however, the use of cell numbers is not recommended as a sole parameter of biomass because Selenastrum capricornutum cell size decreased linearly with an increase in medium concentration. Also, at low cell concentrations or suspended solids levels (< 10 mg/l) the use of suspended solids is not reliable because of the lack of precision at that level.
8. The satisfactory precision (coefficient of variation $\leq 15\%$) of batch assay algal responses obtained in the interlaboratory precision tests

indicate that this test can be recommended as a screening procedure in eutrophication analyses.

NATURAL WATER ASSAYS

9. The batch assay method as described in this report is recommended for the screening of natural waters because linear relations were observed between $\hat{X}$ responses and the concentrations of both Clear Lake and Sacramento River waters, and the $\hat{X}$ and $\hat{\mu}_b$ determinations had an adequate precision (coefficient of variation $\leq 18.5\%$).

10. The use of yield coefficients in batch cultures is not recommended for the identification of growth-limiting nutrients.

11. The measurement of $\hat{\mu}_b$ in wastewater-receiving water mixture assays can identify toxicity which is not detected by $\hat{X}$ responses; therefore, this measurement is strongly recommended as a general algal response measurement when batch assays are used.

12. The use of enrichment (spiking) procedures with batch assays to identify growth-limiting nutrients yielded questionable results in this investigation; however, the method appears to have merit but further research and evaluation are needed before these procedures can be recommended for practical use.

PRESENT STATUS OF THE BATCH ASSAY

13. The batch assay, as described in this report, was inferior in most respects to the chemostat assay and should be used primarily as a screening or monitoring method in eutrophication analyses.

14. Further research must be undertaken on methods to identify the growth-limiting nutrients, to obtain algal growth kinetics of important nuisance algae, and to develop the rational framework necessary to apply batch bioassay results to eutrophication problems in a rational and practical manner.

PRESENT STATUS OF CHEMOSTAT ASSAYS

15. The chemostat assay is the only procedure (method) recommended for determination of any kinetic description of algal growth-nutrient concentration relationships.

16. The chemostat bioassay procedure is highly recommended for determining the concentration of rate-limiting nutrients in natural waters and wastes, the toxic characteristics of wastes, and for identifying the growth rate-limiting nutrients.

17. The use of algal growth kinetic data, and particularly accurately determined values of K_s and $\hat{\mu}$ in combination with enrichment (spiking) tests, is recommended as the most accurate and reliable procedure for identifying the growth-limiting nutrients in natural waters or wastewater mixtures.

18. The most rational application of bioassay results to practical eutrophication problems utilizes kinetic descriptions of the alga and nutrient of concern in conjunction with the results from chemostat assays and nutrient concentration assays on the natural water as described in this report. Its use and further refinement and the research related thereto are strongly recommended.

19. The most urgently needed information in eutrophication analyses today is kinetic descriptions ($\hat{\mu}$, K_s , Y_n , k_d , etc.) for the test alga as well as troublesome bloom algae and the nutrients presumed to be of concern, but particularly phosphorus, nitrogen, carbon, and micronutrients.

20. A biochemical evaluation is recommended strongly to investigate the phenomenon of "excess" nutrient uptake, particularly that of phosphorus with Selenastrum capricornutum and to determine accurately the different forms of phosphorus inside the algal cells and their availability for growth and storage and the kinetics related thereto.

SECTION III

INTRODUCTION

THE PROBLEM OF EUTROPHICATION

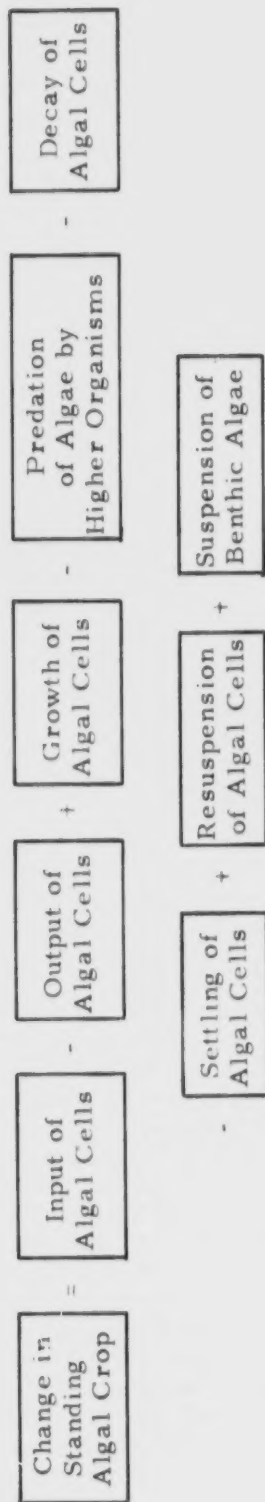
Eutrophication is the natural or artificial enrichment of water systems with nutrients resulting in substantial increases in the productivity and organic matter (mainly algal cells) of the systems. The increased mass of organic matter resulting from the increased productivity frequently limits some of the uses of such water. Activities of man accelerate the addition of nutrients thereby increasing the productivity. Such "cultural eutrophication" may be caused by discharge of domestic wastes, runoff from agricultural operations, wastes from mining and industrial operations, and disruption of natural drainage basins through development, road building, and urbanization. The deleterious effects of cultural eutrophication have been documented for lakes in this country as well as abroad [1-5], and it has been estimated that over half of the surface water supplies in the U. S. are already affected by cultural eutrophication [6]. Stewart and Rohlich [7], the National Academy of Sciences [8], and the Uppsala Symposium [9] have reviewed eutrophication recently.

Effects of Eutrophication

The main effect of eutrophication is an increase in the organic content of the water. In eutrophic waters the development of masses of planktonic algae (primarily blue-green algae) has been the focus of much attention. However, the growth of aquatic plants should not be ignored nor assumed to be negligible [10, 11].

The algal standing crop or concentration in a given water body is a complicated function of many variables. A simplified conceptual description of the algal standing crop in a natural aquatic system is shown in Figure 1. Such a description permits identification of those variables amenable to control by man. Variables which increase the standing crop are the algal growth rate, the concentration of algae in the input water, and the rates of resuspension of planktonic and benthic algae. Variables which decrease the standing crop are the loss of algae in the outflow, sedimentation of algae, and the algal decay rate which includes respiration, mineralization, and consumption by predator organisms.

A constant algal standing crop occurs when the rates of the variables increasing the algal concentration is balanced by the rates of the factors decreasing the algal concentration. An increase in the standing crop, which at noxious levels would constitute a bloom, results when the cumulative rate of the increasing factors exceeds that of the decreasing



$$\frac{dX}{dt} = \frac{F}{V} X_0 - \frac{F}{V} X_1 + \mu_1 (X_1 + X_5) - P_r X_2 \left(\frac{X_3}{X_1} \right) - k_d X_1 - S_s X_1 + S_r X_4 + S_B X_5$$

Where,

- V = Volume of water body, μ = Growth rate of algal mass,
 F = Inflow and outflow rate of water body, P_r = Rate of predation of algal cells,
 X_0 = Algal concentration in inflow water, S_s = Net settling rate of algal cells,
 S_B = Suspension rate of benthic algae, S_r = Resuspension rate of algal cells,
 X_5 = Concentration of benthic algae, k_d = Decay rate of algal cells,

- X_1 = Planktonic algal concentration in water body and outflow,
 X_2 = Concentration of higher organisms responsible for algal predation,
 X_3 = Algal cell concentration available for predation by higher organisms,
 X_4 = Concentration of planktonic algae on the bottom of water body,

FIGURE 1. ALGAL GROWTH MODEL FOR NATURAL AQUATIC SYSTEMS

A decrease in the standing crop, one of the aims of eutrophication control, results when the cumulative rate of the decreasing factors exceeds that of the increasing factors.

Control of Eutrophication

Man can control only some of the factors which affect the level of the standing crop. For example, the rates of predation, sedimentation, and resuspension are very difficult, if not impossible, to control because these are functions of variables such as the algal population composition, higher organism population composition, temperature, wind velocities, etc. The only way to control the algal standing crop at present is to reduce the growth rate of troublesome algae.

The growth rate of algae is controlled by environmental conditions such as temperature and light intensity and also by the concentration of some rate-limiting nutrient (Liebig's law of the minimum) if all nutrients are not in excess. Control of the physical environmental conditions affecting the growth rate is virtually impossible, therefore, control of the algal growth rate can only be effected through reduction of the rate-limiting nutrients present in a specific body of water. The development of methods to remove nutrients from wastes and to determine which wastes require treatment is dependent upon knowing which nutrients control productivity and how the concentrations of the nutrients affect the growth of algae. Therefore it is necessary to understand how eutrophication and the resultant productivity are brought about before rational solutions to the problem are possible.

The lack of understanding of the mechanism of eutrophication is currently exposed in the vigorous controversy over phosphorus, carbon, or nitrogen being the principal limiting nutrient causing algal blooms during cultural eutrophication [12-23]. One popular line of thought is that phosphorus is the principal nutrient involved in causing blooms and it should therefore be removed from at least detergents [14, 19, 20]; however, there are other views being expressed that carbon or nitrogen are the nutrients of concern [15, 17, 18, 22-24].

In general, it is accepted that there is a relationship between dissolved nutrients and the concentrations of algae in a water; also that higher levels of dissolved nutrients result in greater populations of algae. However, few investigators have attempted to use or develop the nutrient concentration - growth rate relationships embodied in fundamental growth models such as reported by Monod [25], Caperon [26], Dugdale [27], Eppley *et al.* [28], and others have used kinetic models to describe growth and nutrient uptake of marine phytoplankton. Smith [29] suggested a similar approach in modeling ecosystems. McGauhey *et al.* [30-33] and others [34] have used kinetic models in describing studies of algal growth in fresh water environments and in evaluating batch and chemostat algal assay studies. In developing rational bases for estimating the allowable concentrations of nutrients in aquatic environments,

such studies are necessary for the specific algae of concern, and particularly for algal species used in standardized algal bioassays.

THE NEED FOR BIOASSAYS

To detect the factors contributing to algal blooms it is necessary to bioassay both wastewaters and receiving waters. The bioassay relates the biological response directly to the water and its nutrient content. Therefore an algal bioassay, as compared to a chemical analysis of the waters, should provide more knowledge of the biostimulatory (or toxic) effects of such waters. Also, a single analysis with a bioassay may suffice to determine gross biostimulant concentrations, whereas a wide variety of chemical analyses would be required to predict the enrichment characteristics based upon the specific nutrient concentrations present.

The need for standard bioassay procedures was recognized by the Joint Industry-Government Task Force on Eutrophication. Candidate methods to measure biostimulatory response were considered at a meeting in Chicago in March 1968 which resulted in a program to develop and/or evaluate a test or tests which could be used in the laboratory to assess quantitatively the algal growth characteristics of a sample of water [35]. Specific criteria for selection of possible procedures were listed at that time; i.e., it was agreed that the assays should have the following characteristics: 1) simple and convenient enough to be performed by technicians, 2) equipment requirements simple and economic, 3) results obtained accurate and reproducible, 4) geographical variation in the method minimal, and 5) results applicable with judgment to the natural environment.

Three candidate tests were suggested for study with reference to the foregoing criteria; the bottle test (batch culture), the chemostat test (continuous flow culture), and an in situ test, the ^{14}C uptake assay (primary productivity).

BIOASSAY OBJECTIVES

Fundamental differences between laboratory bioassays and the field bioassay (^{14}C) required that two different approaches be used. First, a comparative evaluation of the bottle and chemostat tests was to be made and subsequently a field evaluation of the accepted techniques was to be conducted. The general objective of the laboratory evaluation was to compare the two bioassay techniques - batch and continuous flow - for assaying the algal growth and nutrient relationships in natural and enriched waters. Part of the general objective was accomplished as reported in the First Annual Report [34].

The successful application of bioassays to eutrophication control requires the fulfillment of a number of specific objectives as set forth in the First Annual Report. In general, it is required that precise measurement

of algal growth responses be made followed by the application of these responses within a rational framework to obtain the desired results. Therefore, the development and comparison of different bioassay techniques need the co-development of a rational framework in which bioassay results can be applied, with judgment, to solve eutrophication problems.

The development of a rational framework for application of bioassay results must be based on the growth rate-nutrient concentration relationships of algal species of concern. In a specific case the algal species of concern may differ from those of another case. Therefore, the growth rate-nutrient concentration relationships (growth kinetics) of many different algal species must be determined. At the very least, the growth kinetics of the algal species used as test organisms in standardized algal bioassays must be determined to enable interpretation of algal responses in such bioassays.

PREVIOUS ASSAY TECHNIQUES

Many scientists have developed specialized bioassay techniques to deal with specific water and productivity problems. However, there has been little agreement in the data obtained with different techniques and it is difficult, if not impossible, to relate the results obtained from the various methods. Some examples of the different bioassays are discussed to illustrate the diversity of methods used and to indicate possible directions of research on bioassays.

Several investigators have utilized physiological measures as indicators of limiting nutrient concentration and productivity in field samples. Fitzgerald [36], in studies on natural populations of algae and vascular plants, showed that comparative uptake rates for ammonia and measurements of surplus phosphorus or alkaline phosphatase activity can be used to indicate the relative nitrogen or phosphorus availability to the cells. Fitzgerald's [36] results showed that non-nitrogen-fixing algae will take up ammonia at a rate indicating that nitrogen is not readily available. However, in the same environment phosphorus analyses indicated that available phosphorus was in short supply for nitrogen-fixing algae. Therefore, different nutrients can be limiting in the same environment for different species of algae having different requirements. Fitzgerald [36] pointed out the sampling difficulties involved in these bioassays and also indicated some of the important environmental factors, such as rainfall and circulation currents, affected the observed relationships. Eppley and Coatsworth [37] studied uptake rates of nitrate and nitrite and suggested that it may be possible to utilize these results for developing bioassays similar to the ammonia analysis of Fitzgerald [36].

Holm-Hansen in a series of papers, e.g. [38], indicated that ATP, DNA, and organic carbon measurements are useful indices of algal mass. DNA and organic carbon measurements were closely related but did not

distinguish between dead or near dead plant material and live cells as did the ATP test.

Chlorophyll "a" measurements have been used to indicate the activity of algal populations (e. g., Strickland and Parsons [39]). The development of an "in vivo" chlorophyll "a" technique for the analysis of mixed populations of phytoplankton in the field proved to be convenient and sensitive [40]. Because chlorophyll varies with light and physiological state of the algae, measurement of chlorophyll might indicate the state of the cells, that is physiologically active or inactive, as well as providing rough estimates of algal biomass. As an application of chlorophyll measurements, Manny [41] used pigment ratios (chlorophyll "a" and total carotenoids) of fresh water and marine phytoplankton to indicate nitrogen deficiency.

The most commonly used techniques for field bioassays are productivity measurements. The light and dark bottle methods of determining photosynthetic rates (either oxygen production or carbon uptake) have been used for many years, primarily in marine waters but also more commonly in recent years in the fresh water environments. The first productivity measurements were the oxygen bottle techniques of Gaarder and Gran [42]. Later a more sensitive technique was devised by Steemann-Nielsen [43] using the radioisotope ^{14}C and measuring the uptake of $^{14}\text{CO}_2$. This latter method achieved considerable prominence as a means of measuring productivity in situ. Goldman [44] advocated the use of the ^{14}C technique in fresh water environments. Although investigators do not know exactly what phase of algal growth the $^{14}\text{CO}_2$ uptake measures (generally it is considered "net photosynthesis"), it appears to be a convenient and sensitive tool for measuring productivity in natural phytoplankton communities [39] and in determining trace element requirements in fresh water lakes. The in situ analysis suffers from the disadvantage that variations in the algal population sampled may produce results which are difficult to interpret in terms of the biostimulatory response of that water.

Considerable effort and attention has been devoted to laboratory bioassay analysis of water samples. Historically the major method used has been a batch culture assay. However, recently attention has been directed to the use of continuous flow culture bioassays. Both techniques use an essentially constant volume of culture differing operationally only in that no flow occurs in batch assay and a continuous and constant flow of fresh sample occurs in the continuous system. In batch systems the cell and nutrient concentrations change with time while in continuous systems a steady state is attained (see Section IV for further discussion). Because of the traditional use of batch culture assays and their adaptability to the handling of large numbers of samples, batch culture methods have been used extensively to date.

Allen and Nelson [45] first used the batch technique to evaluate the capacity of water samples to grow algae. In recent years investigators

have used either the response of unialgal cultures [46-48] or of mixed algal cultures [49,50] to determine the level of enrichment in water samples. Although unialgal cultures are easier to use, mixed culture advocates believe that a mixed species community approximates the natural situation more closely. Although generally the algal biomass is estimated (weight, numbers, or volume), estimates of algal growth of mixed algal species have been made using chlorophyll fluorescence measurements [50] or ^{14}C accumulation [51].

Frequently the results from batch bioassays of water samples are expressed as maximum cell concentration, X , sometimes called the maximum yield of cells. Although this relationship may be reproducible for a given sample and fixed set of conditions and may give an estimate of the total amount of cells which can grow on the nutrients present in the sample, that value is far removed from the level of algae (standing crop) which occurs in nature. Obviously the fact that one is able to demonstrate growth in excess of that of the natural community in water samples indicates that the maximum cell concentration, X , is rarely if ever attained in nature. Moreover, one would expect that the effects of environmental limiting factors (light, temperature, mixing) and the effects of predation and settling of cells would tend to keep the concentration of algae below the maximum possible level. Since the effects of environmental factors are most frequently expressed in terms of response rates, it is logical that the biostimulatory response be expressed in terms of growth rates also. Response parameters such as maximum growth rate and maximum cell concentrations are being evaluated in ongoing studies at Lake Tahoe to assess biostimulatory response and to determine which parameters are best in terms of accuracy and precision as well as practical interpretability [30-34]. However, no definitive results have been obtained to date. One of the major reasons for the inconclusive results appears to be the inherent variability of batch assays.

Use of the chemostat, a continuous flow culture device, as a bioassay tool has been relatively limited. In the chemostat, cultures are maintained at steady state where all factors are at a steady state, i.e., nearly a constant level. Thus the determination of growth constants is facilitated through the use of cultures in balanced growth. The merit of the chemostat for nutrient-growth relationships was reviewed by Pearson [52] and the results of bioassays of natural waters in the Lake Tahoe Basin and of sewage effluents diluted in these natural waters [30-33,53] have indicated that it should be evaluated thoroughly for use as a routine laboratory assay. Pearson *et al.* [53] have described some comparative bioassays of various waters using batch and chemostat techniques and have suggested that the batch technique produces deceptive results. Borchardt in ongoing work is using the turbidostat (the same fundamental theory as the chemostat) to study natural water samples [54].

Application of results obtained from laboratory bioassays of samples collected from natural waters or waste effluents will always be limited by the degree to which the bioassay models reflect what happens in nature. As soon as a sample is removed from its normal environment one has introduced factors which make that sample nonrepresentative. The effects of light (period and cycle), temperature, the hydromechanics of the aquatic system, exchange of dissolved gases with the atmosphere or benthos, and the presence of decomposers and predators all have been altered because these factors are properties of the environment and are changed simply by the physical fact that the sample is removed from that environment. Therefore, one can only analyze for those properties which are intrinsically a part of the sample, its chemistry (concentration of nutrients and toxicants), and the concentration and composition of organisms, detritus and other particulate matter. Further treatment of the sample such as by filtration to remove particulate matter so that a standard unialgal population may be introduced may only serve to make more difficult the extrapolation of laboratory results for application to nature.

The analyses of field samples suffers from the disadvantage that they measure only what is in the particular sample at the collection time. Also, field bioassays do not allow ready interpretation of the effects of various treatment or of additions of wastewater. Therefore one virtually is forced to bring samples to the laboratory to obtain such information.

Although the basic techniques of laboratory bioassays are similar, very little effort prior to the Provisional Algal Assay Procedure evaluation study has been devoted to standardize methods. Hence, the degree of standardization of algal assays depends in major part on the progress made in this evaluation.

PROVISIONAL ALGAL ASSAY PROCEDURES EVALUATION

To develop and/or evaluate and standardize an algal assay technique many different problems must be investigated. In the evaluation of the Provisional Algal Assay Procedures by the participating groups much attention has been given to the methods for measurement of algal biomass [34, 55-58] and the batch culture assay [34, 55-72]. However, the evaluation of the continuous culture assay method has received relatively little attention [73].

The algal biomass methods that have been studied include measurements of algal cell numbers (hemocytometer counts and electronic particle counts), dry weight, total cell volume, optical density, fluorescence, total carbon, and suspended carbon. In general, good relationships were found between different biomass parameters [34]; however, some measurements appear questionable. Cell numbers appear to be unsatisfactory [34, 55, 61] and fluorescence measurements may be suspect under nitrogen-limited conditions [74]. The use of more than one method during assays is recommended, with all measurements referenced to cell mass on a dry weight basis [73].

The batch assay method has been shown to respond to varying levels of nitrogen and phosphorus [34, 59, 60, 70, 72]. The need for the macro-nutrients, calcium, potassium, and sulfur [60, 70] and carbon [75] was demonstrated, and a sensitivity to iron [70] and other micronutrients (included as a mixture) was also shown [57, 61, 62, 67, 76]. Therefore the inclusion of micronutrients in the reference media was recommended [e.g., 57, 61, 76].

The coefficient of variation of the batch culture assay method appears reasonable at approximately 15% [34]; and similar coefficients of variation (10% - 30%) were observed in an interlaboratory precision test of the batch assay [69]. Carbon-limitation effects were noted in assays of high-nutrient concentration samples [55, 57, 65] resulting in nonlinear responses between concentrations of nutrients (other than carbon) and algal growth [e.g., 55]. Therefore, it was recommended that the pH of the cultures during assays be kept below 8.5 through use of appropriate surface to volume ratio flasks [55] or ventilation systems [34, 55]. Light intensity can influence the growth of the test algae [55]; however, the use of low intensity lighting (150 ft-c) has been suggested [71]. No significant differences were observed between higher light intensities (400 and 800 ft-c) when cell number was the parameter of biomass; however significant differences were observed when carbon content was the parameter of biomass [58].

Nutrient utilization rates in batch assays were found to be influenced by the presence or absence of micronutrients [58], and excess nutrient uptake and initial N/P ratios influenced the organism yield coefficients for nitrogen and phosphorus [34]. Excess uptake of nutrients appears to have occurred in other studies [59, 68]. Inoculum age (between 4 and 35 days) had no apparent effect on organism response in batch assays [58, 61, 71]. Identification of the growth-limiting nutrient in samples in batch assays through the use of enrichment (spiking) techniques succeeded in some cases but not in others [72]. Further study is needed.

The batch assay method has been evaluated extensively. Although the batch assay cannot fulfill all requirements of algal assays, it is expected that a standardized assay method will be recommended in the near future [77]. However, the chemostat assay has not been evaluated and this still remains to be accomplished [73]. Porcella *et al.* [34] and Toerien *et al.* [78] have discussed the application of chemostat assays in eutrophication analysis. The potential dual role of chemostat methods, both as an algal assay method and as a method of obtaining the growth kinetics (growth rate—nutrient concentration relationships) of algae was pointed out [78].

Determination of the growth kinetics of both the algal species used in standardized algal assays and for those algae important in eutrophication is needed to allow interpretation of test organism behavior as well as rational design proposals to solve eutrophication problems. For example, the low half saturation constant (K_s) for phosphorus observed

for Selenastrum capricornutum [34] explains why the growth rates of this organism do not differ significantly from each other under conditions for growth having wide variations in the phosphorus concentration. That is, the phosphorus concentration is so high (excess P) that it is not rate limiting in most instances; hence the growth rate is zero order (independent) of phosphorus concentration in this range. Furthermore, the low values of K_s for phosphorus suggest that for this organism (and types behaving in a similar manner) phosphorus concentrations will have to be extremely low ($< 20 \mu\text{g/l}$) to be the growth rate limiting nutrient in nature. It may not be feasible or possible in many waters to reduce the danger of blooms from these types of organisms by reducing background phosphate concentrations to such levels by domestic waste treatment for phosphate removal.

REPORT OBJECTIVES

The specific objectives of this research investigation and report can be summarized briefly as follows:

1. To evaluate the use of batch culture assays with a standardized, chemically-defined medium.
2. To evaluate the use of batch culture assay of natural samples.
3. To evaluate alternative methods for sample pretreatment.
4. To determine the growth kinetic constants for the test algal species, Selenastrum capricornutum, in a phosphorus-limited environment.
5. To evaluate the use of chemostat assays of natural samples.
6. To compare the relative efficacy of batch and chemostat assays in eutrophication analyses.

SECTION IV

THEORY AND RATIONALE

GENERAL THEORY OF GROWTH OF MICROORGANISMS

Symbols used in the development of kinetic equations in the following pages are based upon the unified concept of fundamental symbols suggested by the "Second International Symposium on Continuous Cultivation of Microorganisms" held in Prague, 1962 [79]. The symbols, their units, and explanations are summarized in Section XI.

Growth of Unicellular Organisms

In the biological sense, growth is an orderly increase of all the components of an organism, not merely of some of its constituents. In all cellular organisms, cell multiplication is a consequence of growth. In unicellular bacterial and algal growth, cell multiplication leads to an increase in the number as well as mass of cells.

Generation Time and Growth Rate

Because growth represents an increase in the amount of living matter, it leads to an increase in self-duplicating material. The more living substance that is formed, the greater becomes the amount of matter that can grow and reproduce itself. Consequently, under ideal conditions for growth and reproduction, the amount of living matter increases not in direct proportion to time, but it multiplies itself by a constant factor in each successive unit of time. The geometric progression with time is illustrated most dramatically by the growth of cultures of most unicellular bacteria and algae. Each individual cell grows, and after reaching a certain size, divides to form two or more complete individuals. In cultures the time of division is randomized, but the time required for each successive doubling of the total population under optimum conditions remains constant. This period of time is called the generation time (g).

Because the number of cells doubles during each generation, the total population increases as the exponent of 2, and such growth is referred to as exponential growth and can be algebraically described as:

$$X = X_0 2^N \quad (1)$$

where

X = concentration of microorganisms at time t , M/V ,
 X_0 = concentration of microorganisms at time 0, M/V , and
 N = number of cell divisions

The following equation

$$\frac{t}{N} = g \quad (2)$$

where

t = time, t ,
 N = number of cell divisions in time interval t
 g = generation time, t

therefore expresses the average generation time or doubling time for the culture as a whole [80]. A rearranged form of Equation 2 can be substituted in Equation 1 and one obtains

$$X = (X_0) \left(2^{t/g} \right) \quad (3)$$

Taking logarithms and rearranging

$$g = \frac{(\ln 2) (t)}{\ln (X) - \ln (X_0)} \quad (4)$$

and solving for X ,

$$\ln (X) = t \frac{(\ln 2)}{g} + \ln (X_0) \quad (5)$$

When the expression $(\ln 2)/g$ reaches its maximum value for a specific organism under specific growth conditions, the maximum specific growth rate, $\hat{\mu}$, can be substituted for $(\ln 2)/g$ as follows:

$$\ln (X) = \hat{\mu}t + \ln (X_0) \quad (6)$$

or

$$X = (X_0) e^{\hat{\mu}t} \quad (7)$$

The equation in this form is recognized as the general growth equation [80]. Taking the derivative with respect to time, the following relationship is obtained

$$\frac{dX}{dt} = \mu X \quad (8)$$

The gross specific growth rate is less than the maximum growth rate when either the substrate is limiting or when conditions are not optimum. The net growth rate is obtained when both the specific growth rate (μ) and organism decay rate (k_d) (i. e., the decrease in cellular mass due to endogenous respiration and cellular death with subsequent lysis) are considered:

$$\frac{dX}{dt} = (\mu - k_d) X \quad (9)$$

Specific Growth Rate as a Function of Limiting Nutrient Concentration

The specific growth rate, μ , is specific for organism, environment, and culture medium. It depends on the growth capacity of the micro-organism and the environment in which it grows. The specific growth rate is a function of the growth rate limiting nutrient concentration when all other factors are in excess. Then

$$\mu = f(S) \quad (10)$$

where

S = growth rate limiting nutrient concentration, M/V

This function is generally expressed in such a way that at low nutrient concentration the specific growth rate is first order with respect to nutrient concentration, whereas at high nutrient concentration, the specific growth rate is zero order with respect to nutrient concentration and it approaches its maximum value, $\hat{\mu}$.

Monod Model (Michaelis-Menten Equation)

The rectangular hyperbola is the model widely accepted in expressing the relationship between specific growth rate, μ , and the rate-limiting concentration. This relationship is based on the Michaelis-Menten equation which was developed to describe the rate of an enzyme reaction as a function of substrate concentration [81] and which was proposed

empirically by Monod [25, 82] to describe the relationship between bacterial growth and substrate concentration:

$$\mu = \hat{\mu} \left(\frac{S}{K_s + S} \right) \quad (11)$$

where

- $\hat{\mu}$ = maximum specific growth rate, time⁻¹
- μ = specific growth rate, time⁻¹
- S = concentration of the growth rate limiting nutrient, M/V
- K_s = half saturation constant, numerically equal to the nutrient concentration at which the specific growth rate is one-half the maximum growth rate, i. e., $\mu = \hat{\mu}/2$.

The Michaelis-Menten (Monod) kinetic model for describing the relationship between the growth rate and nutrient concentration is shown in Figure 2, and this relationship has been widely used by microbiologists and engineers working with continuous culture systems [52, 80, 83-85]. In sanitary engineering, several workers have been successful in using this model for descriptions of activated sludge kinetics, anaerobic fermentation system kinetics, and also algal growth kinetics. Caperon [26], Dugdale [27], Eppley *et al.* [28], and others have used this kinetic model to describe growth and nutrient uptake of marine phytoplankton, McGauhey *et al.* [30-33] have used the same kinetic model in descriptive studies of algal growth in fresh water environments. Porcella *et al.* [34] used the M-M, M equation in evaluating batch and chemostat algal assay studies.

Other Empirical Kinetic Models

Several empirical kinetic relationships have been proposed to describe growth kinetics of microorganisms. However, none of these models had the wide acceptance of the M-M, M kinetic model.

Teissier's Exponential Model

Teissier [86] proposed the following exponential equation to describe the growth rate-nutrient concentration relationship of his bacterial system

$$\mu = \hat{\mu} (1 - e^{-kS}) \quad (12)$$

where

k = a growth constant.

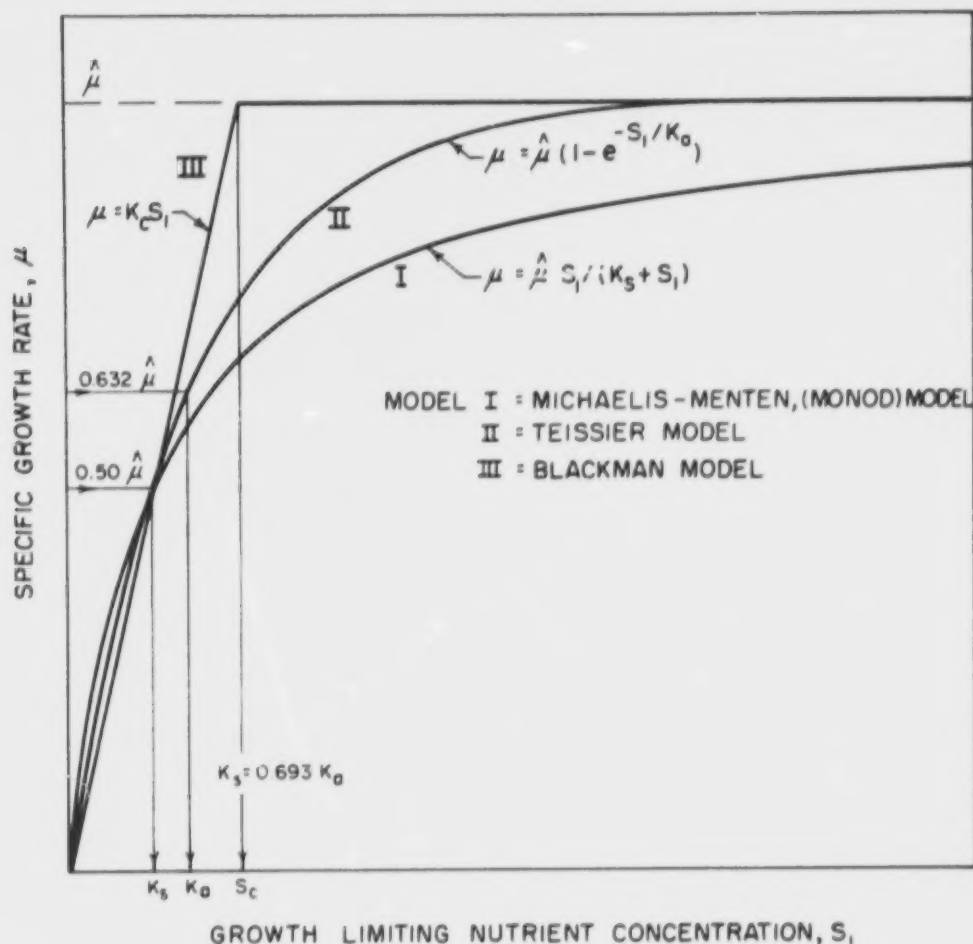


FIGURE 2. A GRAPHICAL COMPARISON OF MICHAELIS-MENTEN, (MONOD) EQUATION, EXPONENTIAL EQUATION AND BLACKMAN MODEL WITH THE SAME VALUES OF MAXIMUM SPECIFIC GROWTH RATE AND HALF SATURATION CONSTANT

Schulze and Lipe [87] and Schulze [88, 89] found that their results fit the exponential equation better than did the M-M, M model. Shelef *et al.* [90] in studies of algal growth kinetics under nitrogen-limiting conditions, suggested a modified Teissier's equation:

$$\mu = \hat{\mu} \left(1 - e^{-S_i / K_a} \right) \quad (13)$$

in which K_a is a growth constant equal to the value of nutrient concentration at a specific growth rate of $0.632 \hat{\mu}$. A graphic comparison of this method is also shown in Figure 2.

Blackman First Order-Zero Order Model

The Blackman first order-zero order kinetic model is based on the assumption that the rate of photosynthesis is proportional to the light intensity or carbon dioxide concentration until a maximum rate is attained at a certain limiting concentration (or light intensity) and thereafter the rate remains constant at the same maximum level regardless of any increase in nutrient concentration or light intensity. This model can be formulated as:

$$\mu = K_c S, \text{ when } \mu < \hat{\mu}, S < S_c$$

$$\mu = \hat{\mu}, S = \alpha, \text{ when } \mu = \hat{\mu}, S \geq S_c$$

where

$$S_c = \text{nutrient saturation concentration, M/V.}$$

A graphic comparison of this model is also shown in Figure 2.

Moser's Model. Moser [91] developed a nonrectangular hyperbola to describe the relationship between growth rate and the concentration of the rate-limiting nutrient as shown in the following:

$$\mu = \hat{\mu} \left(\frac{S^n}{K_s + S^n} \right) \quad (14)$$

In case of $n = 1$, Moser's equation is the same as the M-M, M equation.

Contois' Function. Contois [92] proposed a kinetic equation for description of the specific growth rate in terms of population density, P , as well as the concentration of the limiting nutrient, S :

$$\mu = \hat{\mu} \left(\frac{S}{BP + S} \right) \quad (15)$$

where

B = growth parameter which is constant under defined conditions.

Growth Yield

The cell yield coefficient, Y , is defined as the amount of cell mass produced per unit amount of nutrient depleted in the culture system, and is expressed as,

$$Y = \frac{dX}{-dS} = \frac{\text{Cell mass produced}}{\text{Amount of growth-limiting nutrient removed}} \quad (16)$$

It is generally believed that the yield coefficient is a constant. However, the yield coefficients for nitrogen-limited and phosphorus-limited growth of algae have been shown to be quite variable [34, 93-102]. Generally, yield coefficients are expressed in terms of the rate-limiting nutrient; however, it should be noted that they can be expressed in terms of any constituent of the substrate consumed by the organisms.

GROWTH KINETICS OF MICROBIOLOGICAL SYSTEMS

Ideal Model Reactors

There are three types of systems, classified in terms of flow conditions, which are commonly considered as ideal reactors. These are:

Batch or Nonflow Mixed Reactors. In the typical batch reactor the reactants, viz. nutrients and algae, are charged initially into the reaction vessel, mixed thoroughly, and retained for a period of time during which their interaction proceeds. The end results of the reaction are increased quantities of cells and decreased quantities of nutrients in the nutrient solution. The batch system is a nonsteady state system in which both nutrient and algae concentrations change continuously with time; however, at any given instant these concentrations are uniform throughout the reacting mass.

Plug Flow or Nonback Mix Flow Reactor. In a plug flow reactor each element of the flowing mass follows another in sequence as though separated from it. Consequently, there is no longitudinal mixing between the elements although there may be lateral or radial mixing within each element. Thus a plug flow reactor may be considered as consisting of an infinite train of batch reactors, each having the same residence time as the plug flow reactor itself. Consequently, the kinetics of batch and plug flow reactors are the same.

Continuous Flow Stirred Tank Reactor (CFSTR) or Back Mix Flow Reactor. A CFSTR is a simple homogenous open system consisting of a stirred tank into which growth medium is fed and from which partially expended culture medium emerges at the same rate. The volume is maintained constant by some form of constant level take-off. In the CFSTR the feed rate is maintained at a predetermined level and the effluent containing reaction products (including algal cells) is withdrawn at this same rate. Thus the physiological conditions of cells in the reactor are maintained at a predetermined level and the entire system (nutrients and cells) is at a steady state.

Algal Growth Kinetics in Batch Systems

Batch Growth Curve (Unbalanced Growth). The basic concern with algae in eutrophication is their multiplication or growth. The dynamics of multiplication, as well as of other functions related to it, are the most important aspects of algal growth. Figure 3 represents an ideal batch growth curve in which cell concentration-time, and nutrient concentration-time profiles are superimposed. The algae begin multiplication in a surplus of nutrients, which gradually decreases; however, the single components often are utilized at different rates.

After inoculation of the nutrient medium, some time elapses before the number of algae begins to increase. This period is termed the "lag phase" (Zone 1, Figure 3) and the length of time before cell division depends on the type and age of inoculum (phase of growth of the culture from which the inoculum originated) and the characteristics of the medium. This dependency on the age of the inoculum reflects the wide physiological variation between different growth phases in an ordinary algal batch culture toward the end of the lag phase. The mass of algal cells present in the culture increases considerably without cell division occurring. The cells are preparing for cell division and multiplication. This stage is therefore called the "physiological youth" of the culture, a description which must be taken figuratively, as it is not possible to consider the culture as a single organism. The next stage represents the beginning of multiplication and of the exponential or logarithmic part of the growth curve (Zone 2, Figure 3) in which the algae are multiplying uniformly with an approximately constant growth rate. The rate of growth during this phase is characteristic for any given alga under the particular condition of cultivation and represents the maximal reproductive capacity of that alga in the specific environment. The environmental factors that govern the rate of growth include the nature and concentration of the nutrients, pH, temperature, light, and other physical and chemical variables. The length of the exponential phase differs according to the available amounts of some essential nutrient. However, in a batch culture the period in which the living biophase does not change its properties and grow in a dynamic steady state is generally short and often steady state does not occur at all but is replaced by continuous transition states.

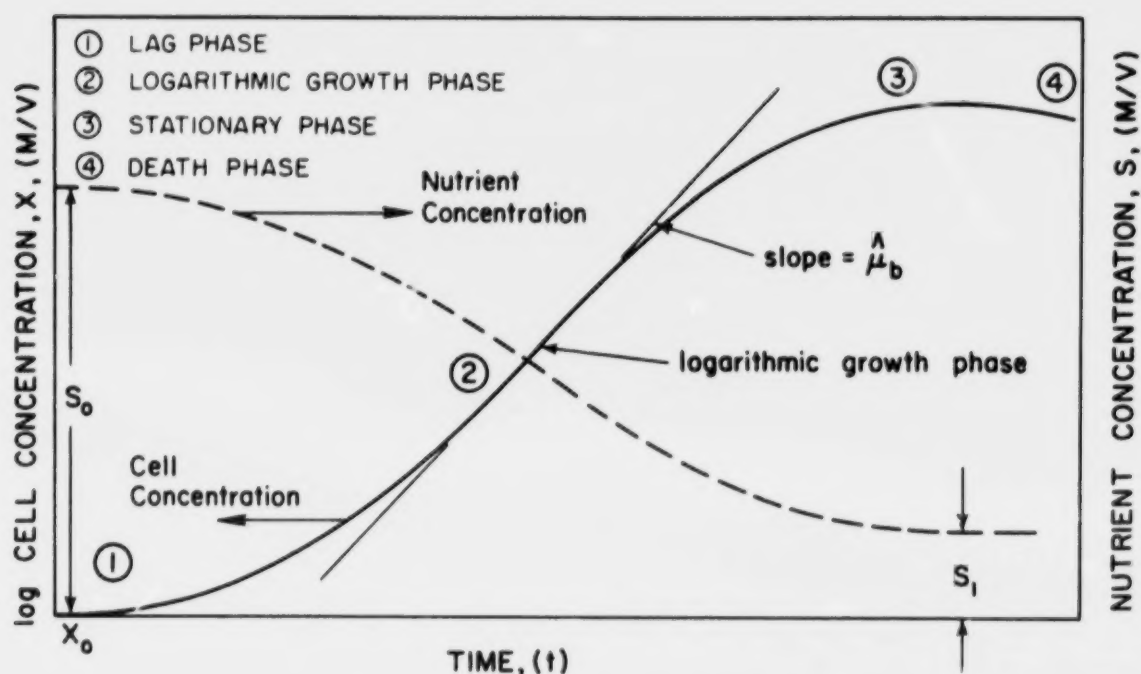


FIGURE 3. IDEAL BATCH GROWTH CURVE

When the algae are sufficiently numerous, their activities begin to have an appreciable effect on the medium. Eventually, some nutrient becomes exhausted, the environment becomes less favorable to growth, and the growth rate decreases to a stationary phase (Zone 3, Figure 3). The stationary phase of some algal cultures passes into a subsequent stage (Zone 4, Figure 3) where the growth curve bends downward as death and lysis of algae in the culture begins to predominate over growth.

The form of the growth curve which is based on the number or the mass of algae does not express completely all changes occurring in such cultures. The algae of one stage of the growth cycle appear to differ physiologically from those of another stage. Therefore, growth in a batch culture is considered to be "unbalanced," i. e., cell concentrations, nutrient concentrations, surface to volume ratios, etc. are changing continually. It has been suggested that the characteristic form of the growth curve and the maximum concentration of cells attainable in a given culture medium reflect the basic natural properties of the algae and their natural population growth characteristics, and on this basis the use of batch culture methods to study these properties has been justified. However, such a concept is erroneous because the basic properties of the algae are affected by the conditions of the

culture method. Therefore, the exclusive use of batch cultures over other methods should not be advocated because of their "unbalanced" growth characteristics.

Evaluation of Batch Growth Kinetics. Mathematical expressions for the kinetics of growth in batch cultures have been presented in the section on the general theory of growth of microorganisms. The growth parameter of batch assays of water samples traditionally is expressed most often as the maximum cell concentration, $\hat{X}$. It is presumed that $\hat{X}$ allows differentiation between samples based on initial nutrient concentration. However, due to difficulties in interpreting what actually limits $\hat{X}$ and in the application of $\hat{X}$ values to practical problems, attention has been directed to the use of other growth parameters, such as maximum specific growth rate batch, $\hat{\mu}_b$; half saturation constants, K_s , for each rate limiting nutrient; and yield coefficient, Y , for each nutrient.

Maximum Cell Concentration, $\hat{X}$. The $\hat{X}$ from batch cultures can be described using the following equation,

$$\hat{X} = X_0 e^{\mu_b t_e} \quad (7)$$

where

t_e = length of time needed to reach zero growth rate

and is measured experimentally as the final cell concentration at the stationary stage of batch growth.

As shown in Equation 7, maximum cell yield varies with initial inoculum, maximum specific growth rate batch, and time to reach zero growth rate. The important concept to remember is that the maximum specific growth rate batch, $\hat{\mu}_b$, is a single function of the growth rate limiting nutrient concentration (Equation 10), while the time needed to reach zero growth is a complex function of all nutrient concentrations and possibly other factors.

Maximum Cell Concentration Limiting Conditions. Batch algal growth in rich "unrestricted" nutrient environments (i. e., all nutrients are considerably in excess) is characterized by the situation where the $\hat{\mu}_b$ approaches $\hat{\mu}$ (largely determined by the environmental conditions of the test) and hence, none of the nutrient species is the growth rate limiting nutrient. This condition is shown schematically in Figure 4. Due to the difficulties of identifying the presumed growth rate limiting nutrient and the possibilities of multiple growth rate limiting conditions, there is no rational mechanism to describe the relationship between

maximum cell production and the concentration of each nutrient species. However, theoretically a maximum cell concentration limiting nutrient is defined as the nutrient species which is exhausted first during the batch growth and presumably has the most significant effect on maximum cell concentration.

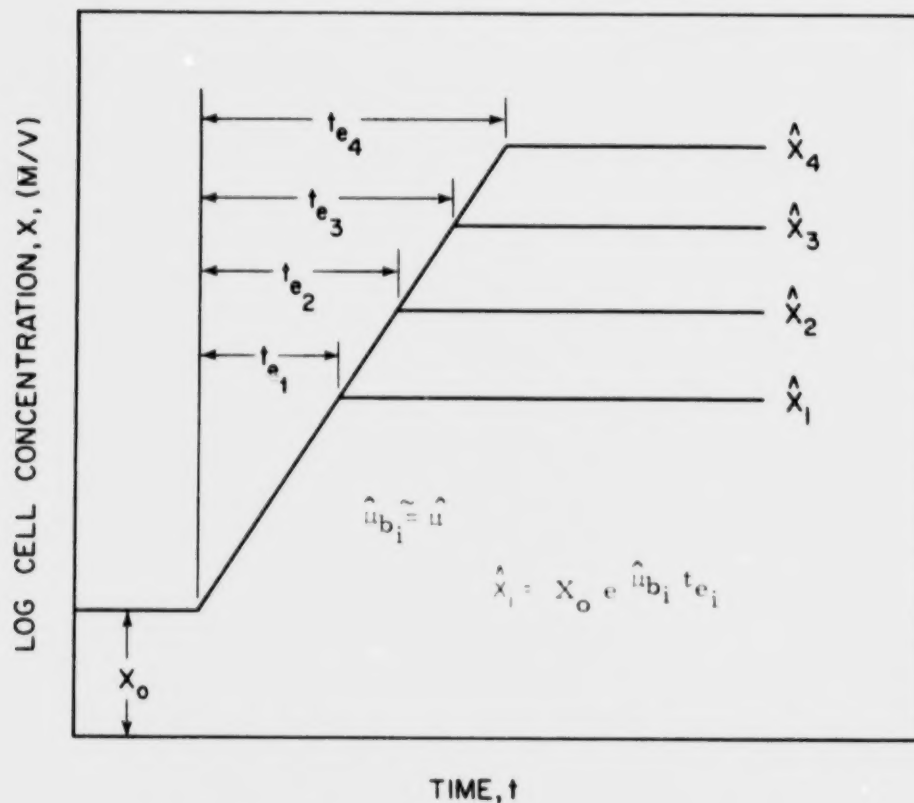


FIGURE 4. BATCH ALGAL GROWTH IN UNRESTRICTED NUTRIENT ENVIRONMENTS

Maximum Cell Concentration and Growth Rate Limiting Conditions. If the algae grow under a specific growth rate limiting condition (i. e., only one specific nutrient limits the growth rate while the other nutrients, growth substances, and growth factors are in excess), the only meaningful growth parameter related to the limiting nutrient concentration is the specific growth rate as indicated in Figure 5. Although the rate-limiting nutrient limits the maximum cell concentration as well, the maximum cell concentration is also related to the length of time needed to reach zero growth rate. This time period cannot be

defined because of its variable character depending upon the characteristics of the sample, the condition of the test alga, and the test method.

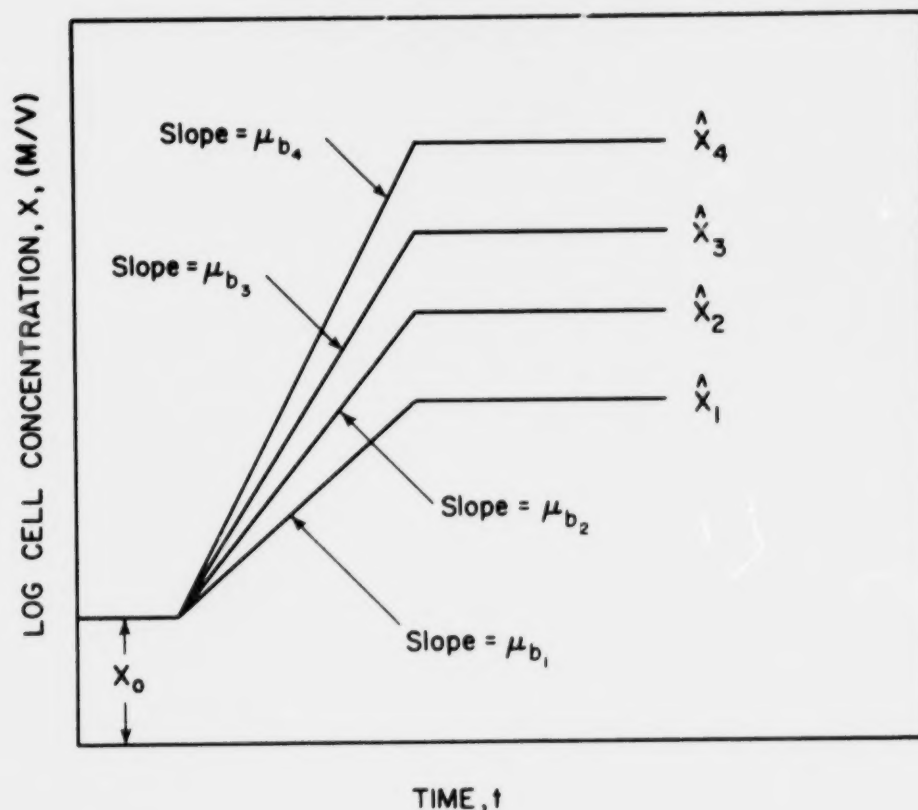


FIGURE 5. BATCH ALGAL GROWTH UNDER SPECIFIC GROWTH RATE LIMITING CONDITIONS

Maximum Specific Growth Rate, $\hat{\mu}_b$. To determine the value of the $\hat{\mu}_b$, Equation 6 can be rearranged as follows:

$$\hat{\mu}_b = \frac{\ln(X_{n+1}) - \ln(X_n)}{t} \quad (17)$$

where

X_{n+1} = cell mass at time $n+1$

X_n = cell mass at time n

t = time interval between exponential growth at time n and $n+1$.

As stated previously, the $\hat{\mu}_b$ is affected by the environmental conditions of the test and is limited by the concentration of the rate-limiting nutrient (Equation 11)

$$\hat{\mu}_b = \hat{\mu} \left(\frac{S}{K_s + S} \right) \quad (11)$$

The specific growth rate batch is equal to the maximum growth rate multiplied by the fraction $S/(K_s + S)$. As K_s is always greater than zero, the value of the fraction is smaller than one and $\hat{\mu}_b$ approaches $\hat{\mu}$ asymptotically as S is increased (i. e., $S \gg K_s$).

To predict the effects of addition of nutrients to and/or removal of nutrients from a water, one must be able to relate the biostimulatory response to the concentration of the specific nutrient. If it is determined that sufficient nutrients are present in the sample to allow $\hat{\mu}_b$ to approach $\hat{\mu}$, it can be concluded that the nutrients are in excess in the natural situation and some other factor(s) (light, CO_2 , temperature, predation, etc.) is limiting the algal population (see Figure 1). On the other hand, if $\hat{\mu}_b \ll \hat{\mu}$, some nutrient is likely limiting growth in the batch culture and may be controlling growth in nature. Consequently, $\hat{\mu}_b$ should be the most interpretable biostimulatory growth response parameter from batch cultivation for specific growth rate limiting conditions.

Algal Growth Kinetics in Continuous Culture (Chemostat) Systems

Continuous flow (chemostat) culture is characterized by the continuous addition of fresh medium (or sample) to the culture, complete mixing of culture and medium, and a continuous outflow of part of the culture at a rate identical to the inflow rate of fresh medium, ensuring a constant culture volume. As long as the chemostat operates, an open dynamic system results in which a steady state is attained. The algal cells in the growth vessel continue to grow at the expense of the fresh nutrients and the total number of algal cells in the chemostat remains constant. Furthermore, the growth rate of the cells can be controlled at will because the slower the fresh medium is delivered to the growth vessel, the slower the cell growth rate. Chemostats provide a method for keeping an algal culture growing for an indefinite period of time, at a constant population size, a controlled and constant rate of growth, a specific, constant physiological state, and constant environmental conditions. It is obvious that the "physiological state" reflects the sum of the biochemical, morphological, and especially physiological features and activities characteristic of the most important factors operative for growth and metabolic activities of the organisms. It was noted in the description of the growth curve for batch cultures that the physiological state often changes rapidly under different environmental

conditions and is the most important characteristic of the state of the culture. However, with the continuous flow (chemostat) culture, culture conditions and the physiological state are maintained essentially constant and at a steady state.

Continuous homogenous cultures can be characterized as complex open systems of constant volume, being as a whole in a dynamic steady state with essentially constant concentrations of all nutrients and of cell biomass. During continuous flow cultivation the biophase permits determination of the kinetics of growth and of growth parameters which can be used in both theoretical analyses as well as for application to practical problems, as will be shown later.

Basic Assumptions. To develop rationally a mathematical model for a continuous flow (chemostat) system, three basic assumptions are made: 1) A constant proportion of the organisms are viable; 2) The (gross) specific growth rate of organisms in a chemostat is some function of the growth rate limiting nutrient concentration, thus

$$\mu = \frac{1}{X} \frac{dX}{dt} = f(S) \quad ; \quad (10)$$

and 3) The specific growth rate of the organisms varies with the rate of consumption of the growth rate limiting nutrient,

$$\frac{dX}{dt} = Y \frac{-dS}{dt} \quad (18)$$

where

Y = cell yield coefficient.

It is generally assumed that the yield coefficient, Y , is a growth constant with respect to any nutrient. However, this is only true when the energy requirement for endogenous respiration for the organism does not affect the constant endogenous metabolism of the organisms at different growth rates, and/or when there is no "excess" uptake or storage of the growth rate limiting nutrient [34, 93-102].

Growth Kinetics in Chemostat with a Constant Yield Coefficient. In developing the growth kinetics of a continuous flow culture system with no cell return and with a constant yield coefficient, the continuity of biomass and nutrients in steady state or the materials balances for biomass and nutrients are developed.

Materials Balance for Cell Biomass. For a CFSTR (Figure 6) a materials balance for the reactor is expressed by the following equation:

$$\begin{aligned} \left[\begin{array}{c} \text{rate of change of} \\ \text{cell biomass in} \\ \text{the reactor} \end{array} \right] &= \left[\begin{array}{c} \text{rate of input} \\ \text{of cells} \end{array} \right] - \left[\begin{array}{c} \text{rate of output} \\ \text{of cells} \end{array} \right] \\ &+ \left[\begin{array}{c} \text{growth rate} \\ \text{of cells} \end{array} \right] - \left[\begin{array}{c} \text{decay rate} \\ \text{of cells} \end{array} \right] \\ V \left(\frac{dX_1}{dt} \right) &= F X_0 - F X_1 + \mu X_1 V - k_d X_1 V \end{aligned} \quad (19)$$

where

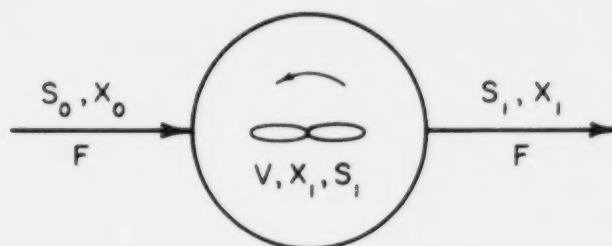
F = flow rate, volume/time
 V = volume of reactor.

At steady state (that is $dX/dt = 0$) in a reactor with no cell recycling and receiving no cells in the input, Equation 19 becomes

$$\mu - k_d = \frac{F}{V} = \frac{1}{\theta} = \frac{1}{\theta_c} \quad (20)$$

where

θ = hydraulic residence time, t
 θ_c = mean cell age in the reactor, t .



Symbols { F = Flow Rate
 X = Cell Mass Concentration
 S = Limiting Nutrient Concentration
 V = Reactor Volume

Subscripts { o = Influent
 i = Effluent or Reactor

FIGURE 6. CONTINUOUS FLOW, STIRRED TANK REACTOR SYSTEM (CFSTR)

Materials Balance for Nutrient. The materials balance of the rate-limiting nutrient through a noncell recycling reactor can be described as follows:

$$\begin{aligned}
 \left[\begin{array}{l} \text{rate of change of} \\ \text{growth rate} \\ \text{limiting nutrient} \\ \text{in reactor} \end{array} \right] &= \left[\begin{array}{l} \text{rate of input of} \\ \text{rate-limiting} \\ \text{nutrient} \end{array} \right] - \left[\begin{array}{l} \text{rate of output of} \\ \text{rate-limiting} \\ \text{nutrient} \end{array} \right] \\
 &\quad - \left[\begin{array}{l} \text{rate of uptake of} \\ \text{rate-limiting} \\ \text{nutrient} \end{array} \right]
 \end{aligned}$$

$$V \frac{dS_i}{dt} = F S_o - F S_i - \text{uptake} \quad (21)$$

The term "uptake" can be defined in terms of the yield coefficient, Y , and the derived form of the natural growth equation (Equation 18). Thus,

$$\frac{dS}{dt} = \frac{dS}{dX} \cdot \frac{dX}{dt} = \frac{1}{Y} \mu X \quad (22)$$

and "uptake" = $\mu X_1 V/Y$.

Therefore,

$$V \frac{dS_1}{dt} = F S_o - F S_1 - \frac{\mu X_1 V}{Y} \quad (23)$$

At steady state

$$\frac{dS}{dt} = 0$$

$$F (S_o - S_1) = \frac{\mu X_1}{Y} \cdot V$$

or

$$X_1 = \frac{F}{V} \frac{(S_o - S_1) Y}{\mu}, \text{ and}$$

$$X_1 = \frac{(S_o - S_1) Y}{\mu \theta} \quad (24)$$

It must be noted that the net yield coefficient ($Y_n = X_1/(S_o - S_1)$) is related to the yield coefficient as follows:

$$Y = \left(\frac{X_1}{S_o - S_1} \right) \mu \theta = Y_n (1 + \theta_c k_d) \quad (25)$$

It can also be shown that the specific growth rate is a function of the rate-limiting nutrient removal velocity, q , which in turn is a function of the rate-limiting nutrient concentration.

$$\mu = \frac{S_o - S_1}{X_1 \theta} Y = Yq \quad (26)$$

where

$$q = \frac{S_o - S_1}{X_1 \theta}$$

Substituting Equation 26 into Equation 20 one obtains the cell continuity equation:

$$\frac{1}{\theta} = \frac{1}{\theta_c} = Yq - k_d = \mu - k_d \quad (27)$$

It should be noted that $1/\theta = 1/\theta_c$ is only true for nonrecycle reactors. Substituting Equation 11 into Equation 27 and solving for S_1 ,

$$S_1 = \frac{K_s \left(\frac{1}{\theta} + k_d \right)}{\hat{\mu} - \left(\frac{1}{\theta} + k_d \right)} \quad (28)$$

Equations 24 and 28 describe steady state values of cell concentration and limiting nutrient concentration, respectively. Inspection of Equation 28 reveals that for nonrecycle reactors where $1/\theta = 1/\theta_c$, once the residence time, θ is selected, the reactor or effluent concentration S_1 has a single value, that is, it is fixed by the residence time as all other terms in the equation are constants. By substituting the value of S_1 from Equation 28 into Equation 24 one obtains the following:

$$X_1 = \frac{Y}{\mu\theta} \left[S_0 - \frac{K_s \left(\frac{1}{\theta} + k_d \right)}{\hat{\mu} - \left(\frac{1}{\theta} + k_d \right)} \right] \quad (29)$$

It should be emphasized that at steady state and for a given residence time θ , and organism, the cell concentration X_1 is a function only of the concentration of nutrient (S_0) in the feed stream. The relationships defined by Equations 24 and 29 are shown graphically in Figure 7. Again it should be noted that for a given residence time, the effluent concentration S_1 , is fixed and independent of the feed concentration S_0 . However, the reactor cell concentration (X_1 , X_2 , and X_3) is determined by feed concentration S_0 , S_{02} , and S_{03} for that residence time. There is a critical value of residence time below which the algal concentration, X_1 , decreases exponentially and finally approaches zero because the residence time is insufficient for the algae to reproduce and maintain the population. Then the nutrient concentration in the chemostat and in the effluent, S_1 , increases until it reaches the influent nutrient concentration, S_0 , because there is no uptake and no algae present. This critical value is referred to as "washout." Using Equations 20 and 27, when θ approaches "washout" (θ_w), μ approaches its maximum value, $\hat{\mu}$, then

$$\frac{1}{\theta_w} = \hat{\mu} - k_d \quad (30)$$

where

θ_w = residence time at which "washout" occurs.

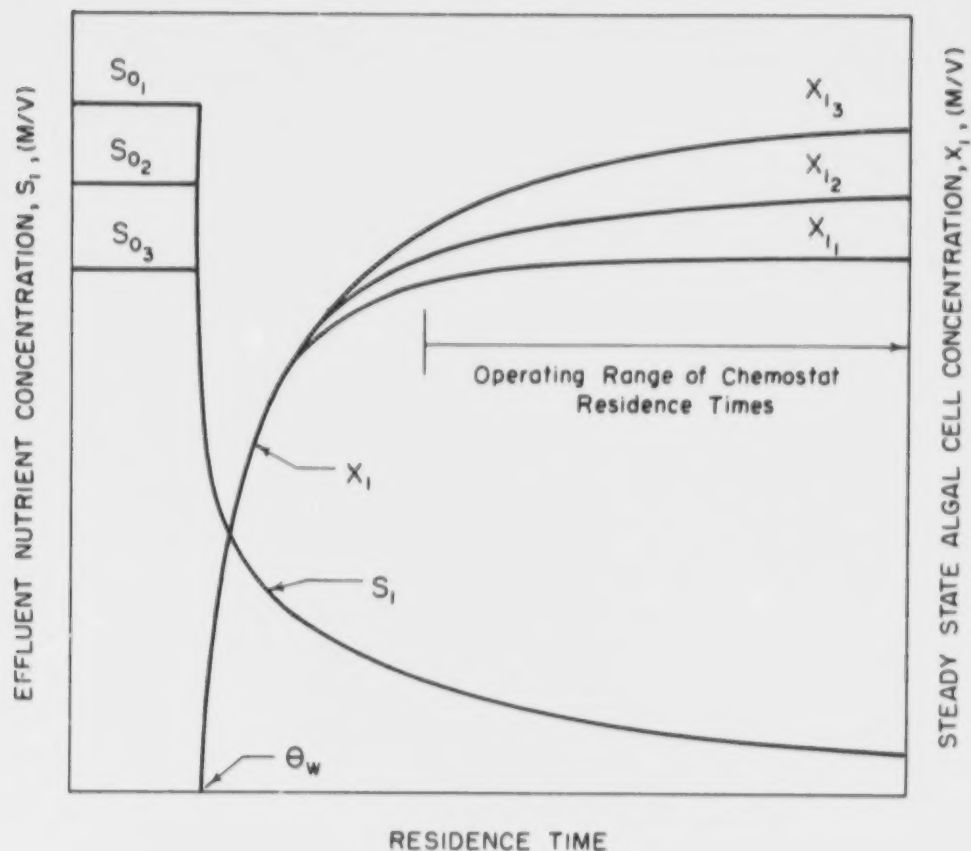
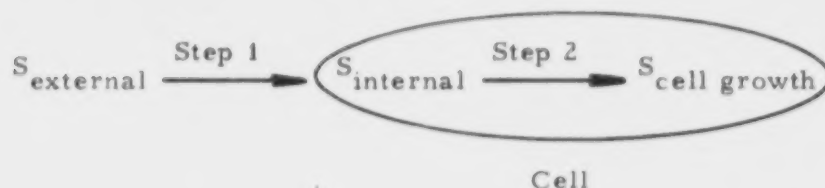


FIGURE 7. THEORETICAL STEADY STATE CONDITIONS IN CHEMOSTAT

Growth Kinetics in a Chemostat with Varying Yield Coefficients. Varying yield coefficients have been observed by many researchers [34, 93-102], and several explanations have been proposed for this phenomenon. Marr *et al.* [99] attributed the varying yield coefficient to the effect of a maintenance requirement for exogenous nutrient by the organisms. Hetling *et al.* [100] attributed the variation of yield coefficient with the dilution rate in chemostat bacteria to the maintenance requirement and presence of dead cells. Mor and Fiechter [101, 102] explained the decrease in cell yield coefficient at low dilution rates as being due to the effect of a constant endogenous metabolism and increased inhibitor formation and concentration. A constant endogenous metabolism becomes a large fraction of the total metabolism when the total metabolism energy requirement is relatively low. In addition, a varying yield coefficient caused by "luxury or excess" nutrient uptake or nutrient storage has been reported by several investigations [96-98].

However, very little is known about the "excess" uptake mechanism or the "excess" nutrient uptake function and its effect on growth kinetics in chemostats.

Nutrient Uptake and Utilization. Two distinct phases can be postulated for the utilization of an environmental nutrient in photosynthetic cell growth; namely, the uptake (removal) of the nutrient from the surrounding medium into the cell and then the subsequent role of the nutrient in the reactions leading to cellular production. This can be summarized as follows:



where

- S_{external} = external nutrient concentration
- S_{internal} = internal nutrient concentration available for cell growth utilization
- $S_{\text{cell growth}}$ = nutrient concentration being utilized for cell growth.

"Excess" or luxury uptake of nutrients is a phenomenon where the rate of nutrient uptake (hereafter called the uptake rate) exceeds the rate at which the nutrient is utilized in growth (hereafter called utilization rate). Consequently the nutrient is accumulated (and presumably stored) in excess to the amount needed for growth.

"Excess" uptake of phosphorus has been observed for various organisms [34, 96-98, 103, 104].

In batch cultures the uptake rate can be estimated from the nutrient depletion with time of growth as shown by Porcella *et al.* [34] for *Selenastrum capricornutum* under conditions of different initial N/P ratios. They found that phosphorus was essentially completely removed by the algae before 50% of the maximum amount of algal growth had taken place, indicating "excess" uptake of this nutrient. They also reported that the wide physiological variations inherent in batch culture systems obscured the relationship between algal growth rate and external and internal phosphorus concentrations.

In continuous culture systems (chemostats), at steady state, all environmental and physiological properties are constant. Then the external phosphorus concentration is regulated by the specific growth

rate of algae or the hydraulic residence time of the system (M-M, M equation). In the case of excess phosphorus uptake the only parameters that would be influenced are the cell concentration and the yield coefficient. Logically, one can expect that at low residence times (low cell age - high growth rate) in chemostats with no cell recycle, the "excess" nutrient taken up by the cells is not completely utilized for cellular growth before the cells leave the system. Therefore, the cell yield coefficient for the specific nutrient is relatively low. However, as the residence time (mean cell age) increases (i. e., growth rate decreases), increasing amounts of the "excess" taken up by the cells are utilized for cellular growth resulting in higher yield coefficients. Finally, when the mean cell age approaches infinity ($\theta_c = \theta \rightarrow \infty$) essentially all of the "excess" nutrient taken up will be utilized for cellular growth and the yield coefficient approaches its maximum value, $Y_{\max}$.

Nutrient Uptake Rate and Nutrient Utilization Rate. The nutrient uptake rate in a chemostat can be defined as the amount of nutrient taken up per unit cell mass per unit time, i. e., the nutrient removal velocity, q :

$$\frac{1}{X} \frac{dS}{dt} = q = \frac{S_o - S_1}{X_1 \theta} \frac{\text{mass nutrient removed}}{\text{cell mass-day}} \quad (31)$$

From the cell continuity equation (Equation 20) the following simple continuity or dimensional analysis can be obtained:

$$\mu = \frac{1}{\theta} + k_d = \frac{1}{X} \frac{dX}{dt} = \frac{dX}{dS} \frac{1}{X} \frac{dS}{dt} = Yq = Y \frac{S_o - S_1}{X_1 \theta} \quad (32)$$

$$\frac{\text{mass of cells produced}}{\text{cell mass-day}} = \frac{\text{mass of cells produced}}{\text{mass of nutrient removed}} \times \frac{\text{mass of nutrient removed}}{\text{cell mass-day}}$$

With a constant yield coefficient, Y , the cells utilize the limiting nutrient for growth at the same rate at which the nutrient is taken up from solution, hence no excess nutrient is accumulated in the cells and the nutrient removal velocity, q , equals the nutrient utilization rate, μ/Y (curve A, Figure 8). However, if "excess" nutrient uptake occurs, the nutrient removal velocity, q , exceeds the utilization rate, $\mu/Y_{\max}$ (curve B, Figure 8). Then,

$$q = \frac{\mu}{Y} = \frac{\mu}{Y_n (1 + \theta_c k_d)} > \frac{\mu}{Y_{\max}} \quad (33)$$

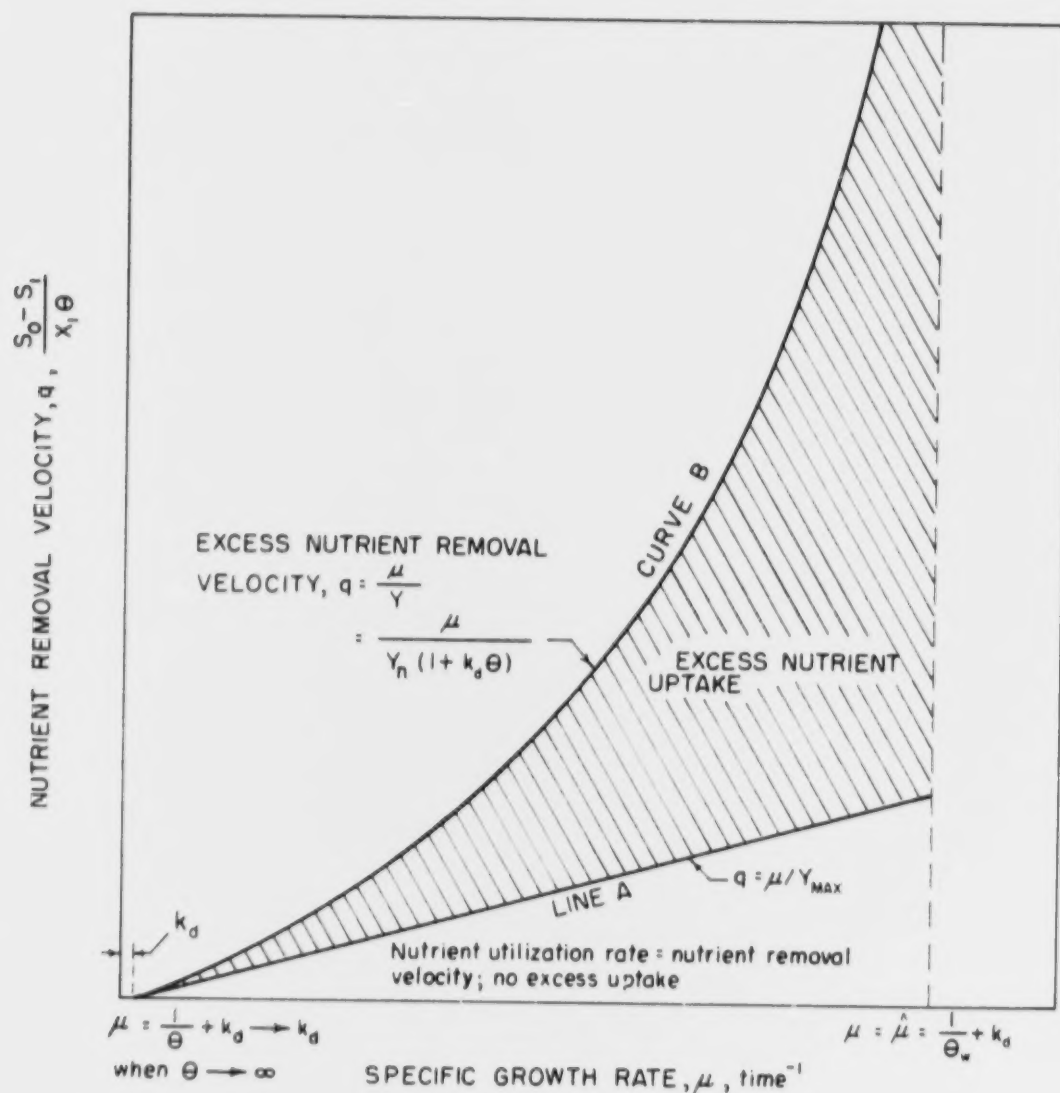


FIGURE 8. COMPARISON OF NUTRIENT REMOVAL VELOCITY AND NUTRIENT UTILIZATION RATE UNDER NO "EXCESS" AND "EXCESS" NUTRIENT UPTAKE CONDITIONS

The shaded area in Figure 8 represents the difference between the uptake rate (with "excess" uptake) and the utilization rate under conditions of no "excess" uptake between the limits of growth rate, $\mu = k_d$ ($\theta = \theta_c \rightarrow \infty$), where no "excess" uptake occurs to a growth rate, $\mu = \hat{\mu}$, where a very high degree of "excess" uptake occurs.

Kinetics of "Excess" Nutrient Uptake in Chemostats. A flow diagram definition sketch for the supply, uptake, and utilization of a specific nutrient for growth in a chemostat at steady state is shown in Figure 9. The nutrient passes through the cell walls and membranes at the nutrient removal rate, q , and is distributed to the functional processes of the cell for cellular synthesis. The nutrient removal velocity, q , in a chemostat is a function of cell age (residence time) and the yield coefficient, i. e.,

$$q = \frac{\mu}{Y} = \frac{\mu}{Y_n (1 + \theta_c k_d)} = \frac{S_o - S_1}{X_1 \theta_c} \quad (34)$$

The nutrient utilization rate constant, k_1 , is a growth parameter specific for the algal species, nutrient, and the environment in which the "excess" nutrient taken up or stored is utilized for growth. In the absence of any theoretical basis for the kinetic model of this reaction, it is assumed to be a first order reaction according to:

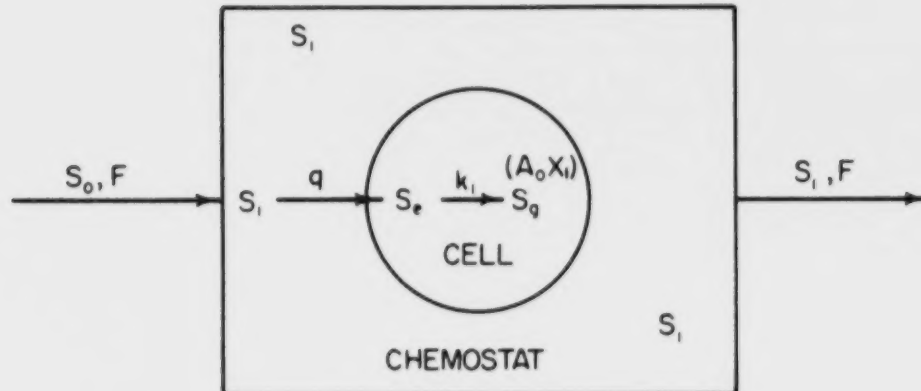
$$\frac{dS_e}{d\theta} = \frac{-dS_g}{d\theta} = -k_1 S_e \quad (35)$$

where

- S_e = nutrient concentration available for cell growth including "excess" nutrient uptake and storage
- S_g = nutrient concentration utilized by cells for their growth, and this amount = $A_o X_1$
- A_o = nutrient content of individual cell at infinite cell age in chemostat = $(S_o - S_1)/X_{max}$
- A = nutrient content of individual cell at cell age θ in chemostat = $(S_o - S_1)/X_1$.

In accordance with a materials balance for the specific nutrient in the chemostat system, the following equation must hold:

$$\begin{aligned} \left[\begin{array}{c} \text{nutrient} \\ \text{supply} \end{array} \right] &= \left[\begin{array}{c} \text{extracellular nutrient} \\ \text{remaining in} \\ \text{chemostat} \end{array} \right] + \left[\begin{array}{c} \text{intracellular nutrient} \\ \text{available for} \\ \text{cell growth} \end{array} \right] \\ &+ \left[\begin{array}{c} \text{nutrient content of} \\ \text{individual cell} \\ \text{at } \theta = \infty \end{array} \right] \times \left[\begin{array}{c} \text{steady state cell} \\ \text{concentration at} \\ \text{cell age } \theta_c \end{array} \right] \\ S_o &= S_1 + S_e + A_o X_1 \quad (36) \end{aligned}$$



A_0 = NUTRIENT CONTENT OF INDIVIDUAL CELL AT INFINITE CELL AGE IN CHEMOSTAT, $\frac{S_0 - S_1}{X_{max}}$

A = NUTRIENT CONTENT OF INDIVIDUAL CELL AT HYDRAULIC RESIDENCE TIME, θ , $\frac{S_0 - S_1}{X_1}$

S_e = NUTRIENT CONCENTRATION AVAILABLE FOR CELL GROWTH INCLUDING EXCESS NUTRIENT UPTAKE AND STORAGE

X_{max} = MAXIMUM CELL CONCENTRATION IN CHEMOSTAT AT INFINITE CELL AGE

S_q = NUTRIENT CONCENTRATION UTILIZED FOR GROWTH BY CELL

FIGURE 9. DEFINITION SKETCH FOR A SPECIFIC NUTRIENT SUPPLY, UPTAKE AND UTILIZATION FOR GROWTH IN A CHEMOSTAT AT STEADY STATE

The differential equation (Equation 35) can be integrated between two limits; from $\theta = 0$, $X_1 = 0$, to $\theta = \theta$, $X_1 = X_1$, with a discontinuity when $\theta \leq \theta_w$ as shown in Figure 10. When $\theta = \theta_w$ all the cells are washed out from the chemostat

$$\ln S_e \left| \begin{matrix} S_e \\ S_e \text{ at } \theta = 0 \end{matrix} \right. = \ln (S_0 - S_1 - A_0 X_1) \left| \begin{matrix} X_1 = X_1 \\ X_1 = 0 \end{matrix} \right. = -k_1 \theta \left| \begin{matrix} \theta = \theta \\ \theta = 0 \end{matrix} \right.$$

$$\ln (S_0 - S_1 - A_0 X_1) - \ln (S_0 - S_1) = -k_1 \theta$$

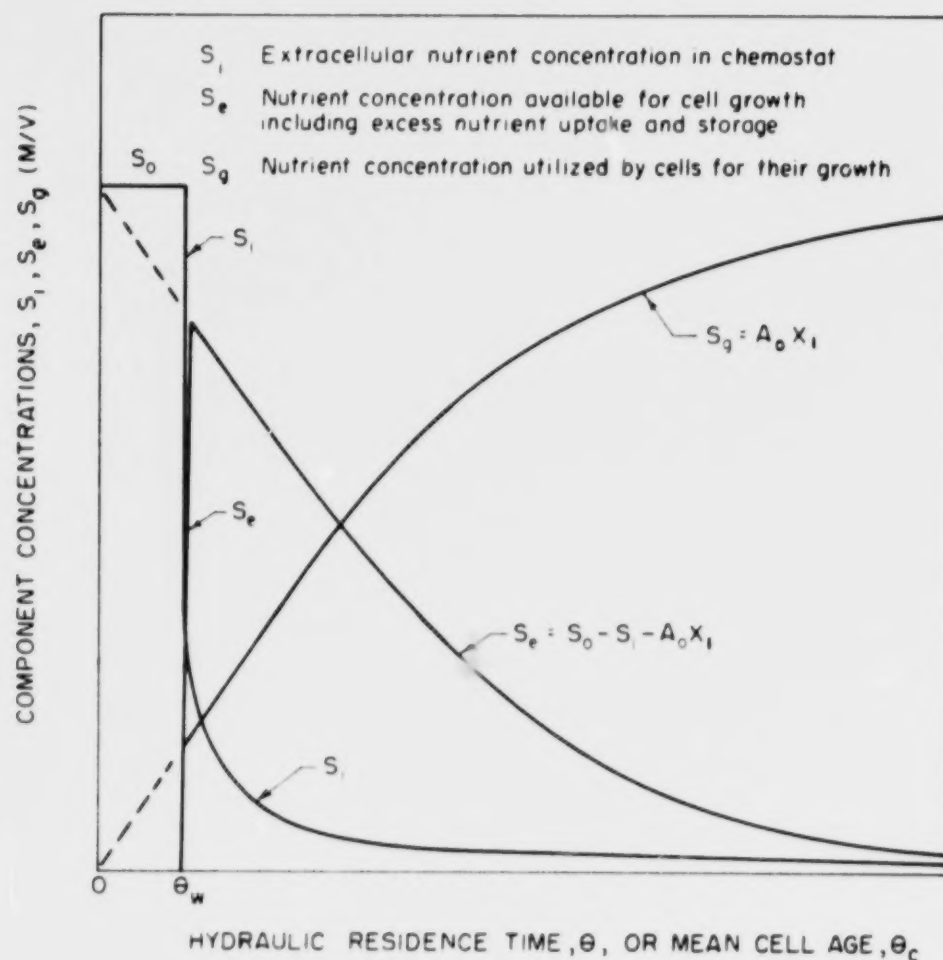


FIGURE 10. COMPONENT NUTRIENT CONCENTRATIONS FOR VARYING MEAN CELL AGES FOR A SPECIFIC NUTRIENT IN STEADY STATE CHEMOSTAT OPERATION WHEN "EXCESS" NUTRIENT UPTAKE OCCURS

Since $A_0 = (S_0 - S_1)/X_{\max}$ and substituting

$$(S_0 - S_1) - \frac{(S_0 - S_1) X_1}{X_{\max}} = (S_0 - S_1) e^{-k_1 \theta} \quad (37)$$

Rearranging Equation 37, the following simple relation is obtained to describe the cell concentration, X_1 , in a chemostat in terms of the maximum steady state cell concentration, $X_{\max}$, for a specific nutrient supply to the chemostat.

$$X_1 = X_{\max} \left(1 - e^{-k_1 \theta} \right) \quad (38)$$

It is apparent that the net yield coefficient, $Y_n = X_1 / (S_0 - S_1)$, varies exponentially with the hydraulic residence time (mean cell age) in a chemostat without cell recycle.

$$\frac{X_1}{S_0 - S_1} = Y_n = \frac{X_{\max}}{S_0 - S_1} \left(1 - e^{-k_1 \theta} \right) = Y_{\max} \left(1 - e^{-\theta_c / K_A} \right) \quad (39)$$

where

$$Y_{\max} = \text{maximum cell yield coefficient} = X_{\max} / (S_0 - S_1)$$

$$K_A = \text{nutrient utilization constant corresponding to the reciprocal of the nutrient utilization rate, i. e., } K_A = 1/k_1.$$

The distribution of the nutrient components, S_1 , S_e , and S_g , as a function of hydraulic residence time, θ , (no cell recycle) or mean cell age, θ_c , in a chemostat is shown in Figure 10.

Empirical Growth-Yield Function. Fuhs [96] proposed empirically that for two marine diatoms, Cyclotella nana and Thalassiosira fluvialis, the relative specific growth rate, $\mu/\hat{\mu}$, is related to the relative amount of phosphate per cell, A/A_0 , as follows:

$$\frac{\mu}{\hat{\mu}} = \left(1 - 2^{(1 - A/A_0)} \right) \quad (40)$$

where

$$A = \text{amount of phosphorus per cell}$$

$$A_0 = \text{minimum amount of phosphorus per cell.}$$

In terms of the cell yield coefficient, Fuhs' equation can be rearranged as,

$$Y_n = Y_{\max} \left(\frac{\ln 2}{\ln 2 - \ln (1 - \mu/\hat{\mu})} \right) \quad (41)$$

The equation of Fuhs (Equation 40) empirically describes the relationship between μ and intracellular phosphorus concentration for two marine diatoms. On the other hand, Equation 39 is proposed for the general description of the phenomenon of "excess" nutrient uptake in chemostats in terms of a varying yield coefficient which is a function of mean cell age.

Rectangular Hyperbola Yield Function. Because of the general acceptance of the rectangular hyperbolic functions (e. g., the M-M, M model) for relating growth rate and limiting-nutrient concentration, a rectangular hyperbolic function appears to be logical for the relation between the cell yield coefficient and mean cell age as follows:

$$Y_n = Y_{\max} \left(\frac{\theta_c}{\theta_c + K_B} \right) \quad (42)$$

where

K_B = a nutrient utilization constant corresponding to the mean cell age at $Y_n = Y_{\max}/2$.

A graphical comparison of the exponential function (Equation 39) and the rectangular hyperbolic function (Equation 42) for a specific value of $Y_{\max}$ is shown in Figure 11.

Fraction of "Excess" Nutrient Uptake, f_e . The amount of "excess" nutrient uptake, S_e , can be described based on Equations 36 and 39 follows:

$$S_e = S_o - S_1 - A_o X_1 = (S_o - S_1) \left(1 - \frac{X_1}{X_{\max}} \right) \quad (43)$$

$$= (S_o - S_1) \left(e^{-\theta_c/K_A} \right) \quad (44)$$

Equation 44 indicates that the amount of "excess" nutrient uptake is proportional to the amount of total nutrient uptake, $S_o - S_1$, and varies exponentially with the hydraulic residence time. The fraction of "excess" nutrient uptake, f_e , defined as the fraction of the total nutrient uptake which is accumulated and/or stored inside the cell (not yet utilized for growth), $S_e/(S_o - S_1)$, is a simple function of mean cell age or hydraulic residence time. This can be shown as follows:

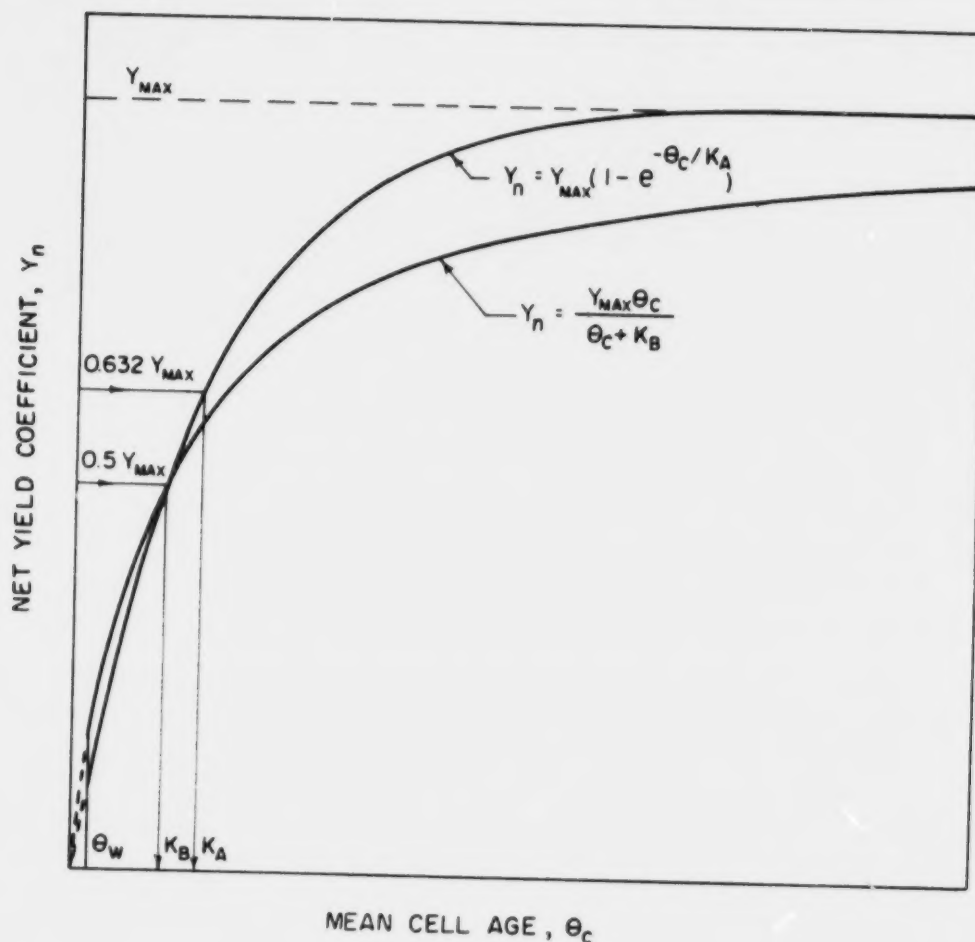


FIGURE 11. GRAPHICAL COMPARISON OF EXPONENTIAL FUNCTION AND RECTANGULAR HYPERBOLIC FUNCTION FOR NET YIELD COEFFICIENT - CELL AGE RELATIONSHIP (SAME VALUE OF MAXIMUM CELL YIELD, Y_{MAX})

As an exponential function based upon Equation 39,

$$f_e = \frac{S_e}{S_o - S_l} = 1 - \frac{X_l}{X_{max}} = e^{-\theta_c/K_A} ; \quad (45)$$

As a rectangular hyperbola based upon Equations 42 and 43,

$$f_e = \frac{S_e}{S_o - S_1} = 1 - \frac{\theta_c}{\theta_c + K_B} = \frac{K_B}{\theta_c + K_B} \quad (46)$$

Equations 45 and 46 are shown graphically in Figure 12.

Chemostat Kinetic Equations with a Varying Yield Coefficient. Kinetic equations for the chemostat with a varying yield coefficient caused by "excess" nutrient uptake can be summarized as follows:

Exponential Model. Cell continuity equation:

$$\frac{1}{\theta} = \frac{1}{\theta_c} = Y \cdot q - k_d \quad (27)$$

where

$$q = \frac{S_o - S_1}{X_1 \theta}$$

$$Y = Y_n (1 + \theta_c k_d) \quad (25)$$

Cell yield coefficient - mean cell age equation:

$$Y_n = Y_{\max} \left(1 - e^{-\theta_c / K_A} \right) \quad (39)$$

where

$$Y_n = \frac{X_1}{S_o - S_1}$$

$$Y_{\max} = \frac{X_{\max}}{S_o - S_1}$$

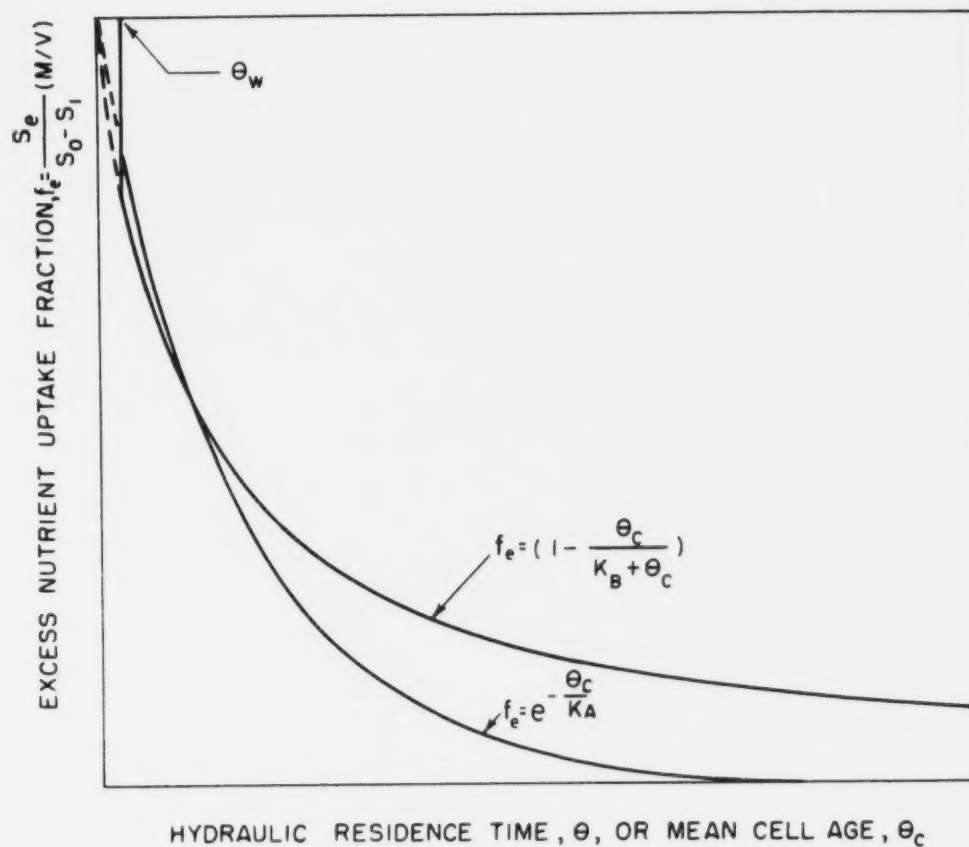


FIGURE 12. THE FRACTION OF EXCESS NUTRIENT UPTAKE AS A FUNCTION OF MEAN CELL AGE (COMPARISON OF EXPONENTIAL AND HYPERBOLIC MODELS)

Specific growth rate limiting nutrient concentration relationship;

$$\mu = \hat{\mu} \left(\frac{S_1}{K_s + S_1} \right) \quad (11)$$

where

$$\mu = \frac{1}{\theta} + k_d \quad (20)$$

$$\hat{\mu} = \frac{1}{\theta_w} + k_d \quad (30)$$

Steady state cell concentration;

$$X_1 = (S_0 - S_1) Y_{\max} \left(1 - e^{-\theta_c / K_A} \right) \quad (47)$$

Rectangular Hyperbolic Model. Cell continuity equation:

$$\frac{1}{\theta} = \frac{1}{\theta_c} = Y \cdot q - k_d \quad (27)$$

Cell yield coefficient - mean cell age equation

$$Y_n = \frac{Y_{\max} \theta_c}{\theta_c + K_B} \quad (42)$$

where

$$Y_n = \frac{X_1}{S_0 - S_1}$$

$$Y_{\max} = \frac{X_{\max}}{S_0 - S_1}.$$

Specific growth rate limiting nutrient concentration relationship is:

$$\mu = \hat{\mu} \left(\frac{S_1}{K_s + S_1} \right) \quad (11)$$

where

$$\mu = \frac{1}{\theta} + k_d \quad (20)$$

$$\hat{\mu} = \frac{1}{\theta_c} + k_d \quad (30)$$

Steady state cell concentration is:

$$X_1 = (S_0 - S_1) \frac{Y_{\max} \theta_c}{\theta_c + K_B} \quad (48)$$

Evaluation of Chemostat Growth Kinetic Constants. The general biostimulatory growth parameters of major concern in the chemostat are: steady state cell concentration, X_1 ; maximum specific growth rate, $\hat{\mu}$, the half saturation constant, K_s ; the nutrient utilization constants, K_A and K_B ; and the decay rate of the organism, k_d .

Steady State Cell Concentration in Chemostat, X_1 . Analogous to $\hat{X}$ of batch cultures, X_1 is the steady state cell concentration attainable in a chemostat for a specific residence time and nutrient concentration in the feed stream. The steady state cell concentration reflects directly the biological available nutrient concentration as shown by Equation 29 which is graphically portrayed in Figure 7. Even when "excess" nutrient uptake occurs, the steady state cell concentration, X_1 , reflects directly the biologically available nutrient concentration in accordance with Equation 47 which is graphically illustrated in Figure 13. It may be noted from Figures 7 and 13 that for a given residence time, the cell concentration X_1 is only dependent on the nutrient concentration, S_0 , in the feed. However, the kinetic description for X_1 in terms of S_0 has different parameters for the case of "excess" nutrient uptake (Equation 47) as compared to no "excess" uptake (Equation 29).

Maximum Specific Growth Rate, $\hat{\mu}$. The $\hat{\mu}$ of a specific algal species determined by chemostat assays is a growth constant in the M-M, M equation (Equation 11) and is closely related to "washout" residence time, θ_w , (Equation 30). This value represents the maximum growth rate of an algal culture and corresponds to the $\hat{\mu}_b$ value of a batch culture under unrestricted conditions, i. e., under conditions where no nutrient or factor is limiting growth. The maximum specific growth rates of algal species are important growth parameters affecting the characteristics and composition of natural biological systems (e. g., they determine which species will grow in a given system as well as the distribution of species based on the competitive ability of particular species for the rate-limiting nutrient in the environment).

Half Saturation Constant, K_s . The half saturation constant is defined as the growth rate limiting nutrient concentration which can support algal growth at one-half the maximum specific growth rate ($\hat{\mu}/2$) and it is specific for each organism and growth rate limiting condition (Figure 2). It is the most important growth parameter in growth kinetics as well as in the application of growth constants to practical problems. The practical significance of K_s will be discussed in a subsequent section.

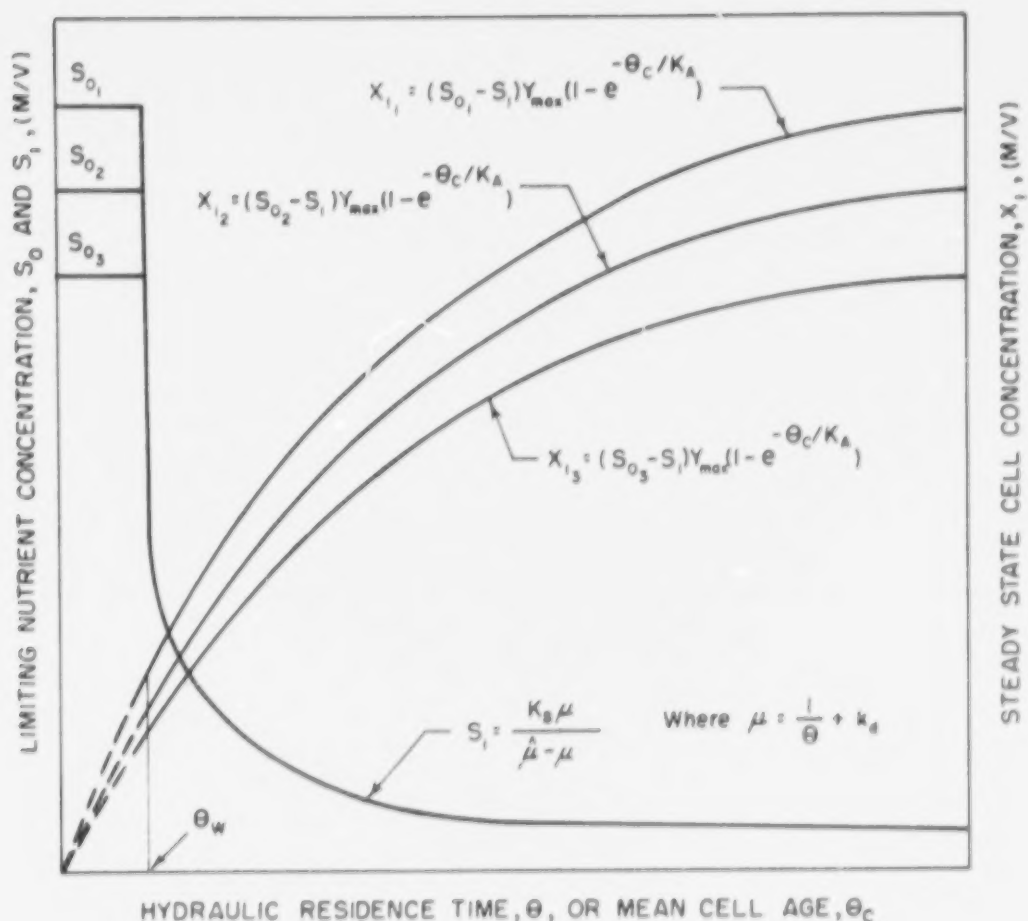


FIGURE 13. THEORETICAL STEADY STATE CONDITIONS IN CHEMOSTAT WITH A VARYING YIELD COEFFICIENT (EXCESS NUTRIENT UPTAKE)

Nutrient Utilization Constants, K_A and K_B . The nutrient utilization constant, K_A , is the mean cell age at which the net yield coefficient (Y_n) is equal to 0.632 maximum yield coefficient, i. e., $Y_n = 0.632 Y_{max}$ in the exponential yield model. Similarly, the nutrient utilization constant, K_B , is the mean cell age at which the net yield coefficient is one-half the maximum yield coefficient in the rectangular hyperbolic model (Figure 11). The nutrient utilization constants represent the rate of nutrient utilization for growth relative to the nutrient removal rate for a specific nutrient, algal species, and

environmental conditions. The higher the value of nutrient utilization constants (K_A or K_B), the lower the nutrient utilization rate (e. g., $k_1 = 1/K_A$) and an increasing amount of "removed" nutrient is accumulated and stored inside the cell. With no "excess" nutrient uptake, the nutrient utilization constants will be zero and the net yield coefficient approaches a constant value equal to Y_{max} .

Decay Rate, k_d . The decay constant, k_d , for an organism reflects the rate of decrease in cellular mass due to endogenous respiration and cellular death with subsequent lysis. The magnitude of k_d might be expected to vary from about zero to values as high as 0.05 day^{-1} (i. e., $0 \leq k_d \leq 0.05 \text{ day}^{-1}$). Frequently the decay rate is ignored or neglected regardless of its possible significant effect on the net cell growth rate. The significance of the decay rate, k_d , is at present not known and because of its small magnitude many investigators apparently choose to neglect it in kinetic analyses. However, from a theoretical and complete analytical standpoint, the decay rate must be included in the materials balance equations. Only by its inclusion and laboratory evaluation of its magnitude will it be possible to understand its role and significance in algal culture systems.

SECTION V

METHODS AND MATERIALS

ORGANISM SELECTION AND RELATED FACTORS

Algal Species

In selection of the algal species to be used in algal bioassays, consideration was given in the Provisional Algal Assay Procedures [35] to the characteristics required from such an organism. It was recognized that the algae should have a broad nutrient response, a distinct shape, and a uniform size. It was further deemed necessary that it should divide distinctly, not attach to glass or surfaces, stay in suspension with slight agitation, not clump, grow at the maximum rate in a medium simple to constitute, be nonauxotrophic, not excrete toxic substances, and normally be associated with oligotrophic waters. These considerations led to the selection of Selenastrum capricornutum Printz as a test organism. This alga has cells which are characteristically lunate with apices acutely pointed; cells are approximately 2 μ wide and 20 μ long and it occurs in both oligotrophic and eutrophic waters [48]. The distinct morphology, little form variation, the fact that cells occur mostly singly and stay in free suspension, and its obligate autotrophic nature make this organism a superior test alga. For these reasons cultures of Selenastrum capricornutum were selected for use in the work described in this report. These cultures provided initially by O. Skulberg (NIVA, Oslo, Norway) were obtained from a common stock culture maintained by T. E. Maloney of the Pacific Northwest Water Laboratory, Corvallis, Oregon. The culture of S. capricornutum was plated several times to eliminate possible algal contaminants and to minimize bacterial contamination.

Nutrient Medium

The nutrient medium outlined in the Provisional Algal Assay Procedures [35] was used as basic nutrient medium. The medium is designated 100% PAAP and its macronutrients are listed in Table I and the micronutrients in Table II. The reagents used were ACS reagent grade chemicals.

Inoculum Preparation

A 7-day old culture of S. capricornutum was centrifuged and the supernatant discarded. The sedimented cells were resuspended in distilled water so that when this suspension was added to the test flasks it resulted in the required inoculation concentration of 1000 cells/ml.

TABLE I
MACRONUTRIENT COMPOSITION OF 100% PAAP MEDIUM

Components ^a	Concentration mg/l	Essential Nutrient	Concentration mg/l
NaNO ₃	85.00	N	14.00
K ₂ HPO ₄	3.48	P	0.62
MgCl ₂	19.00	Mg	9.68
MgSO ₄ · 7H ₂ O	49.00	S	6.37
CaCl ₂ · 2H ₂ O	14.70	Ca	4.01
Na ₂ CO ₃	50.00	K	1.56
FeCl ₃	0.32	Fe	0.11
Na ₂ EDTA · 2H ₂ O ^b	1.00		

^a Before addition of iron and trace metals the pH was adjusted to 7.0 to minimize precipitation.

^b Na₂EDTA = Disodium ethylene diamine tetra acetic acid.

TABLE II
MICRONUTRIENT COMPOSITION OF 100% PAAP MEDIUM

Component	Concentration µg/l	Essential Nutrient	Concentration µg/l
H ₃ BO ₃	618.40	B	110.00
MnCl ₂	880.88	Mn	380.00
ZnCl ₂	109.03	Zn	50.00
CoCl ₂	2.60	Co	1.18
CuCl ₂	0.03	Cu	0.01
Na ₂ MoO ₄ · 2H ₂ O	24.20	Mo	9.60
Na ₂ EDTA · 2H ₂ O	7440.		

Note: The trace metals and EDTA were combined in a single stock mix at a level of 1000 times the final concentration.

Culture Environment

Both batch and chemostat experiments were conducted in a constant temperature room ($24^{\circ}\text{C} \pm 1^{\circ}\text{C}$) equipped with "cool white" fluorescent tube lighting to provide a light intensity of 400 ft-c (4300 lux) $\pm 10\%$. Light intensity for the chemostats was measured adjacent to the reactor at the top, middle, and bottom of the liquid column at points on the reactor nearest to the light source. For batch tests, the light intensity was measured adjacent to the flask at the liquid level.

The pH was maintained in the chemostats between 7.0 and 8.5 by aerating from the bottom with air or an air- CO_2 mixture. The CO_2 content of the air was not maintained at a fixed concentration but was varied as necessary to maintain the desired pH range. The pH in batch tests was maintained in the same range (7.0 to 8.5) by ventilation with air or air- CO_2 mixtures.

WATER SAMPLES

Four different water samples were used in this study. Samples were collected when needed and were not stored for long periods before sample pretreatment was carried out.

Clear Lake Water

The Clear Lake water samples were taken from the pump inlet at the Clear Lake Mosquito Abatement Research Laboratory. The inlet was located about 50 yd offshore in an area where no septic tank outlets or other sources of waste discharges were located.

Sacramento River Water

The samples of Sacramento River water were taken off the river bank about a half mile upstream of the bridge at Knights Landing at the California Department of Water Resources sampling station.

Lake Anza Water

The Lake Anza water samples were taken at the outflow weir in Tilden Regional Park, Berkeley.

Secondary Effluent

Secondary effluent samples from a pilot plant treating domestic sewage from the City of Richmond at the Sanitary Engineering Research Laboratory, University of California were used.

SAMPLE PRETREATMENT METHODS

Several different sample pretreatment methods were evaluated in this investigation.

Autoclaving

The water samples were autoclaved at 121°C for 15 min, cooled down to approximately 25°C, and used in assays.

Pasteurization

The water samples were pasteurized by heating to 71°C (in some cases boiling point) for approximately 10 sec followed by cooling down to 25°C before use.

Filtration

Depending on the assay, samples were either filtered through Whatman No. 41 filter paper or GF/C glass fiber filters or through Millipore membrane (0.45 μ) filters. If necessary, pressure was used to improve filtration rates.

ANALYTICAL METHODS

Biomass Analyses

Biomass was determined by the following four methods:

Absorbance. Absorbance was measured against a distilled water blank at 600 m μ in a one inch cell in a B and L Spectrometer 20. Results were expressed as OD/inch.

Suspended Solids. The determination of suspended solids was made by use of preweighed glass fiber filters (GF/C) which had been previously rinsed with distilled water. Samples of known volume were filtered, the filters dried at 105°C and then placed in individual desiccator jars. Suspended solids in mg/l were determined by difference after 24 hr.

Cell Counts and Total Cell Volume. Cell counts were determined with a Coulter counter Model B and total cell volume estimates were obtained using a mean cell volume computer (Coulter Electronics). Results were expressed as cell number/ml and $\mu^3 \times 10^6$ /ml, respectively.

Nutrient Analyses

Nitrates and Nitrites (Soluble). Nitrates were determined using the cadmium reduction technique according to Strickland and Parsons [105]. The nitrate is first reduced to nitrite when the sample is run through a column containing amalgamated cadmium filings. The nitrite thus produced plus about 95% of the original nitrite present in the sample is determined by diazotizing with sulphanilamide and then coupling with N - (1-naphthyl) ethylenediamine to form a highly colored azo dye, the extinction of which is measured at the wavelength of 543 mμ.

Organic Nitrogen. Organic nitrogen was determined by distillation and Kjeldahl techniques according to Standard Methods [106].

Ammonia. Ammonia was determined with the colorimetric method of Solorzano [107] measuring the blue color of indophenol after the reaction at high pH of ammonia, phenol and hypochlorite.

Orthophosphates (Soluble). Orthophosphates were determined according to Strickland and Parsons [105]. The sample after filtration is allowed to react with a composite reagent containing molybdic acid, ascorbic acid, and trivalent antimony. The resulting complex heteropolyacid is reduced in situ to give a blue color, the extinction of which is measured at 885 mμ.

Total Phosphorus. Before determination of total phosphorus an appropriate volume (10 - 40 ml) of the unfiltered sample was digested with 9 ml of HNO₃ and 1 ml of H₂SO₄ in 100 ml Kjeldahl flasks until the white fumes of NO₂ gas disappeared. The volume of sample remaining was diluted and the pH value adjusted to about 4 with Na₂CO₃. After the sample volume was brought up to 100 ml, the determination was made as for orthophosphates.

Precision and Accuracy of Analyses. The precision and accuracy of analyses were given by Porcella et al. [34] except that the coefficient of variation of orthophosphate analyses at extremely low concentrations was reduced by exerting extreme caution when conducting the analyses. The coefficient of variation for cell counts and cell volume estimates by use of an electronic particle counter was also determined. The values of these coefficients for the orthophosphates, cell counts, and cell volumes, as well as those previously reported, are summarized in Table III.

TABLE III
ACCURACY AND PRECISION OF ANALYTICAL METHODS
AND BIOMASS ESTIMATES

Analysis	Mean	Coefficient of Variation ^a (%)
pH	7.98	0.5
Absorbance OD/one-inch cell	0.026 0.113 0.33	5.1 1.9 0.9
Hemocytometer cell counts Cells/mm ³	823 1420 2010	14.8 9.9 8.3
Coulter counter cell counts Cells x 10 ⁶ /ml	17.3 118.3	3.4 1.6
Total cell volume $\mu^3 \times 10^6$ /ml	1.6 12.7	3.2 1.3
Suspended solids mg/l	9.41 16.5 74.0	7.8 1.3 3.1
Suspended carbon mg/l	4.68 17.7 58.7	13.3 1.7 1.1
NO ₃ $\mu\text{g/l as N}$	10.7 1520 3970	13.5 4.1 2.2
PO ₄ $\mu\text{g/l as P}$	0.7 5.1 75.5 303	20.9 7.6 4.8 1.4
Total P $\mu\text{g/l as P}$	135 561 840	14.0 2.0 6.9

^a Coefficient of variation (%) = 100 (standard deviation)/(mean).

ASSAY TECHNIQUES

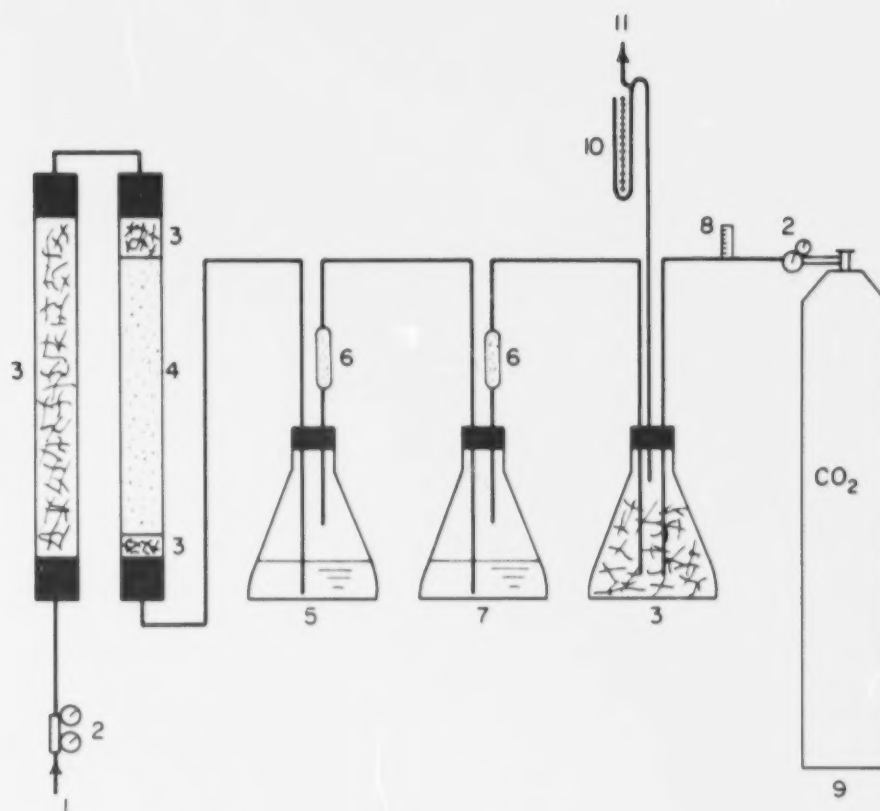
Bottle or Batch Culture Assays

Pyrex glass 500 ml Erlenmeyer flasks were used for batch culturing. The flasks were cleaned carefully according to the Provisional Algal Assay Procedures [35], and 250 ml quantities of either media or natural water samples were transferred to individual flasks. The media or water were inoculated with Selenastrum capricornutum and the flasks were closed with 150-ml Pyrex beakers (inverted) and connected to the ventilation system. This ventilation system could utilize either air or an air-CO₂ mixture (CO₂ in concentrations as needed). The gas was filtered through glass wool and activated carbon and was then passed through an acid solution and distilled water successively before being distributed to each flask as shown in Figure 14 (see also Appendix A). The rate of gas flow to each chemostat or to the flasks was regulated through in-line rotameters. The gas flow was regulated to maintain the pH in the flasks between 7.0 and 8.5. The flasks connected to the ventilation system were then clamped onto a rotary shaker (New Brunswick Scientific Co., Model VS) and shaken at 120 oscillations/min as shown in Figure 15.

In general, an initial cell count and cell volume determination were made on each flask immediately following inoculation. Thereafter counts and volume estimates were made daily from the second to the eighth day of incubation to allow estimates of the maximum specific growth rate, batch (μ_b) of the algae. When the stationary phase was reached in the cultures (14 to 21 days) a terminal cell count, cell volume, and suspended solids determination were made on each flask. Suspended solids measurements were not used to determine μ_b because the μ_b values are obtained early in an assay (first to third day), at which time the suspended solids determinations are extremely imprecise due to low values (<5 mg/l). In addition to the above, the initial and terminal nutrient concentrations in the natural samples were determined to allow estimates of yield coefficients.

Continuous Culture (Chemostat) Assays

Chemostat Design. A schematic diagram of the chemostat algal cultivation system is shown in Figure 16. The chemostat reactor was made of Pyrex glass with flat glass bottom, the top closed by a "café-au-lait" stopper, a siphon break was provided in the input line, and it was provided with magnetic stirring. In the feeding system, glass tubing was used whenever possible, minimum lengths of Tygon tubing were used for joints. Each chemostat was aerated with an air-CO₂ mixture at an air flow rate of approximately 0.5 ft³/hr; the specific flow rate depending on the amount necessary to maintain the pH in the



- | | |
|---------------------------------------|------------------------------|
| 1 AIR SUPPLY | 6 GLASS BEAD TRAP |
| 2 REGULATORS | 7 DISTILLED H ₂ O |
| 3 GLASS WOOL | 8 ROTAMETER |
| 4 ACTIVATED CARBON | 9 CO ₂ SUPPLY |
| 5 0.1N H ₂ SO ₄ | 10 MANOMETER |
| 11 TO GROWTH UNITS | |

FIGURE 14. AIR TREATMENT AND DISTRIBUTION SYSTEM

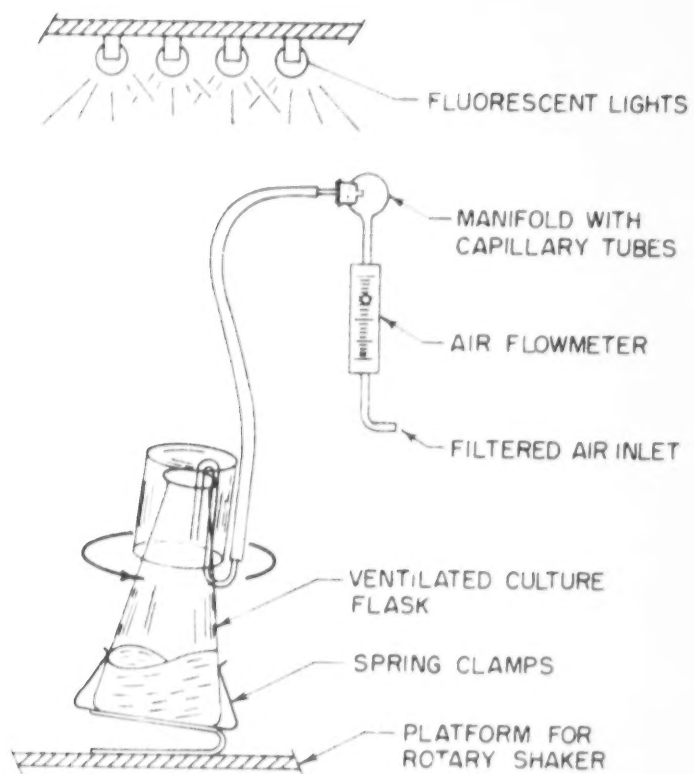
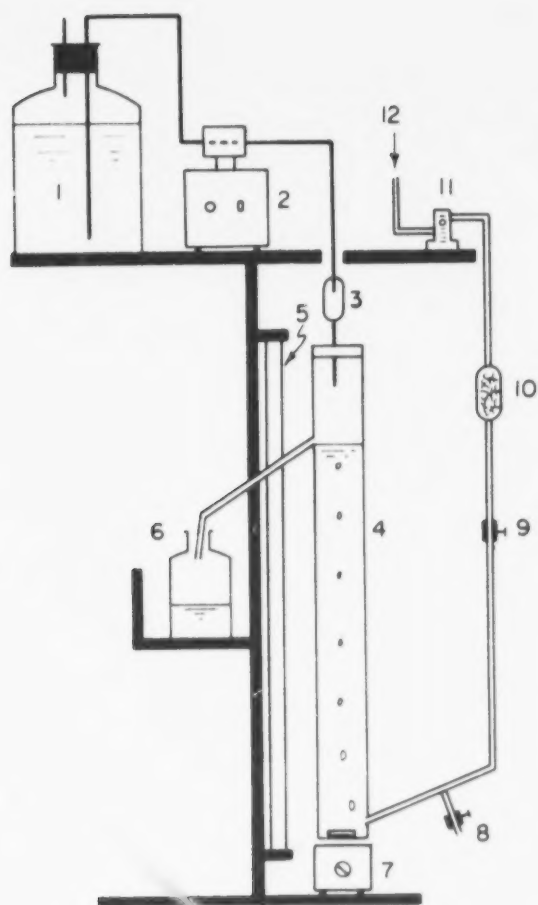


FIGURE 15. BATCH ASSAY UNIT



- | | |
|---------------------|--------------------------------|
| 1 FEED BOTTLE | 7 MAGNETIC STIRRER |
| 2 POLYSTALTIC PUMP | 8 SAMPLING POINT |
| 3 SIPHON BREAK | 9 AIR FLOW RATE CONTROL |
| 4 CHEMOSTAT REACTOR | 10 COTTON WOOL AIR FILTER |
| 5 FLUORESCENT LIGHT | 11 ROTAMETER |
| 6 EFFLUENT BOTTLE | 12 AIR - CO ₂ INPUT |

FIGURE 16. DIAGRAM OF CHEMOSTAT ASSAY UNIT

cultures between 7.0 and 8.3. The pH can be controlled by altering the air flow rate (within the range of 0.1 to 1.0 ft³/hr) or by altering the amount of CO₂ in the air. CO₂ was added as steady state was approached and thereafter during steady state only aeration at 0.5 ft³/hr was used. The air was filtered through an acid and water wash after passing through activated carbon and glass fiber columns (Figure 14).

Chemostat Operation. The chemostats, including all tubing and the medium, were autoclaved at 121°C for 15 min. The chemostats were then filled with the sterilized medium, inoculated, and the cultures allowed to grow before a constant feed rate was established. The chemostats were operated at a constant feed rate for at least two hydraulic residence times. After this period the cultures were operated until "steady state" conditions were established.

After the cell mass concentration in the chemostat had reached its initial maximum value, the attainment of a near constant cell mass concentration implied that steady state had been reached. "Steady state" for this investigation was defined as a period of at least one residence time wherein the cell mass concentrations, determined at daily intervals, did not vary more than $\pm 20\%$ from a mean value. This variation was to be random, i.e., the cell mass concentration could not exhibit a consistent upward or downward trend. In general the tests were continued for 3 or more residence times before the steady state values were obtained.

To minimize volume changes inside the chemostats, only 20-ml samples were withdrawn daily from the reactors. Analyses of pH, absorbance, and cell counts were made on the reactor samples. All steady state analyses of biomass (absorbance, suspended solids, and cell counts) and nutrients were conducted on 100-ml samples withdrawn from chemostats when steady state was reached. The volume of the effluents were collected and measured daily to ensure constant residence times during the entire assay period. The complete analyses scheme is shown in Figure 17.

Computational Methods. A computer linear least square regression analysis was used to estimate the kinetic growth constants for all linear kinetic equations and all linear transformations from nonlinear equations. A nonlinear computer program (COMMON FILE CAL G2 NLIN, Appendix B) was also used to estimate the constants directly from nonlinear regression analyses.

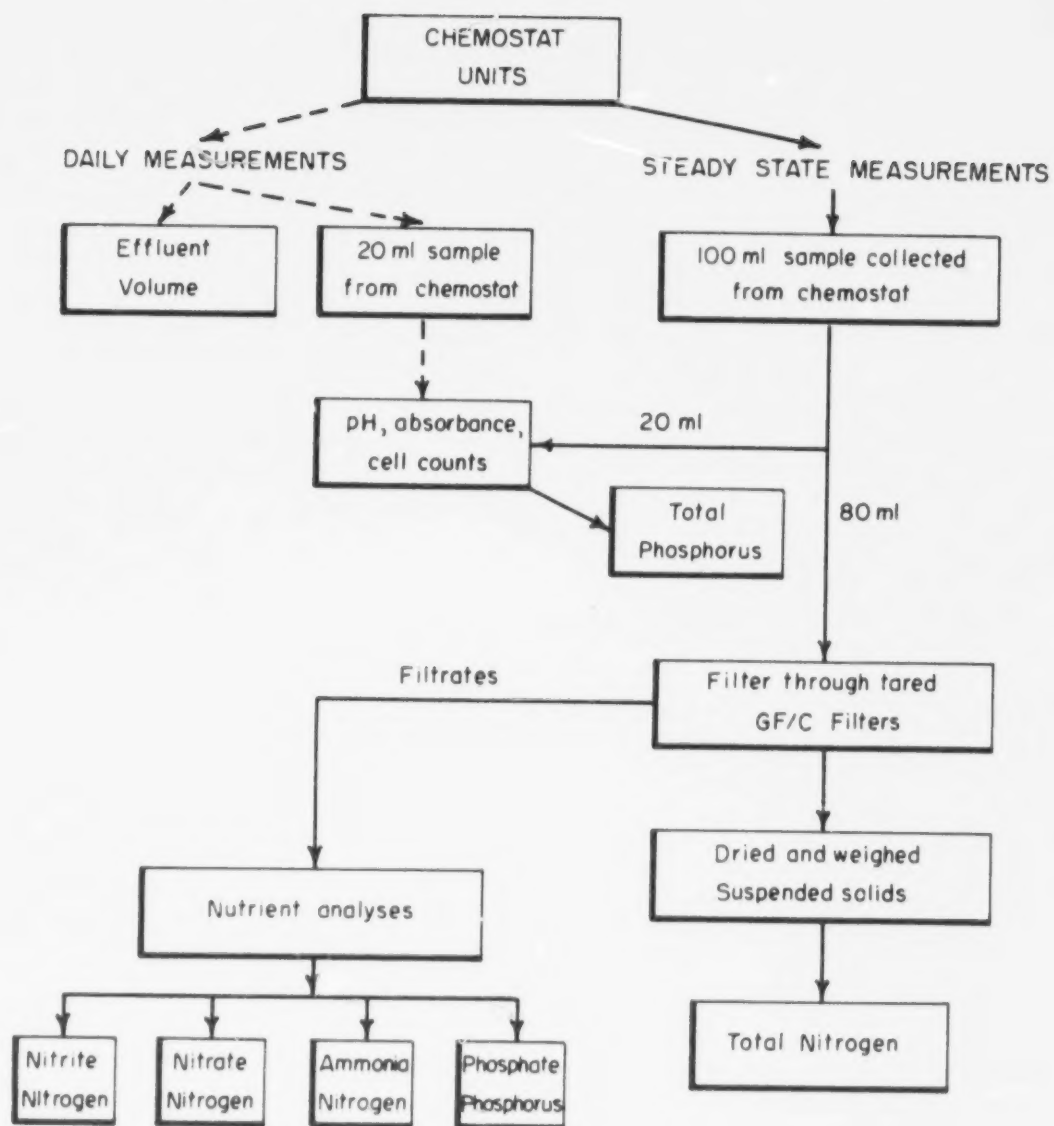


FIGURE 17. FLOW DIAGRAM OF ROUTINE ANALYSES PERFORMED ON CHEMOSTATS

SECTION VI

RESULTS AND DISCUSSION

BATCH CULTURE STUDIES

Sample Pretreatment Evaluation

In accordance with a decision of the PAAP evaluation group meeting in Corvallis, Oregon in May 1970 [108], four different sample pretreatment methods, i. e., autoclaving, pasteurization, and two filtration techniques were evaluated. Each treatment was evaluated for its 1) ability to remove indigenous algae from samples, 2) effect on the nutrient concentrations in the sample, and 3) effect on the algal response in batch assays.

Removal of Indigenous Algae. To simulate a natural water, an aliquot from a high-rate algal pond was spiked into 100% PAAP medium (Tables I and II) in a proportion of 1 to 99, respectively. Samples (250 ml) of this mixture were treated by different pasteurization and filtration techniques (100°C for 10 sec, 71°C for 15 sec). The samples were then incubated under standard PAAP conditions [35] while ventilating the cultures as described by Porcella *et al.* [34]. The absorbance of the cultures were determined initially and at termination 12 days later.

The absorbance values (Table IV) showed that although the control flasks had a tenfold increase in absorbance, none of the pasteurized or filtered samples showed growth. Therefore, it may be concluded that algae were removed effectively from the PAAP medium by pasteurization and filtration techniques.

To determine if indigenous algae of a natural water sample would also be removed by the pretreatment methods, a grab sample from Lake Anza, (Berkeley, California) was used. This sample was halved and one-half was spiked with high-rate algal pond water (1 to 99 ratio) to ensure the presence of algae. Both the spiked and the nonspiked waters were divided into 4 aliquots and subjected to the pretreatment methods, i. e., autoclaving, pasteurization (71°C for 15 sec), and filtration through Whatman No. 41 and membrane (0.45- μ pore size) filters. The resulting waters were transferred aseptically to batch assay flasks, using three replicate flasks for each treatment. The flasks were incubated (without inoculation) under standard PAAP conditions [35] for three weeks. Two cell counts, using an electronic particle counter, were performed during incubation (12th and 21st days).

TABLE IV
REMOVAL OF ALGAE FROM PAAP MEDIUM BY
PASTEURIZATION AND FILTRATION

Treatment	Initial Absorbance	Terminal Absorbance (12 days)	Observation
Pasteurization 71°C for 15 sec	0.015	0.015	No growth
Pasteurization 100°C for 10 sec	0.015	0.015	No growth
Filtration Whatman No. 41	0.015	0.015	No growth
Filtration Membrane, 0.45 μ	0.015	0.015	No growth
Control	0.015	0.16	Growth

The results are summarized in Table V. An untreated control indicated that the maximum count obtainable in the water was approximately 145×10^3 particles/ml. Except for Whatman filtration, none of the other procedures approached this value. However, interpretation of the results is difficult due to a high initial background count in the samples (approximately to 60×10^3 particles/ml). The lower values for the heat-treated samples suggest that these treatments removed the indigenous algae, however, the values for membrane filtration samples indicate that this method was only partially effective in removing the indigenous algae. Whatman filtration treated samples showed the highest values, hence Whatman filtration does not appear to be effective in removing indigenous algae. It is noteworthy that membrane filtration through 0.45 μ membranes was found by Dixon [109] to be unable to remove a small alga from marine samples.

The high background counts obtained with the electronic particle counter is probably due to debris normally associated with natural water samples. Because the electronic counter does not distinguish between debris and algal cells, such counts are not reported as algal counts but rather as particle counts. This shortcoming limits the usefulness of these instruments, particularly in assays of low-nutrient concentration natural samples. Under these circumstances, microscopical observations to verify the presence or absence of debris is necessary.

TABLE V
REMOVAL OF INDIGENOUS ALGAE FROM A
NATURAL SAMPLE BY PRETREATMENTS

Pretreatment Method	Algal Spiking ^a	Counts x 1000/m ³ ^b	
		12th day	21st day
Pasteurization 71°C for 15 sec	Not spiked	42.3 (85%) ^c	32.5 (40%)
	Spiked	69.6 (58%)	34.1 (2%)
Autoclaving	Not spiked	19.0 (123%)	34.7 (154%)
	Spiked	11.5 (39%)	14.3 (95%)
Filtration Membrane, 0.45μ	Not spiked	41.2 (78%)	107.0 (65%)
	Spiked	40.0 (138%)	56.6 (103%)
Filtration Whatman No. 41	Not spiked	62.5 (13%)	101.9 (25%)
	Spiked	86.8 (16%)	162.9 (19%)
Control	Not spiked	146.2	145.9

^aOne part high-rate algal pond water to 99 parts sample.

^bMean of 3 replicates.

^cValue in parentheses = coefficient of variation = standard deviation (100)/mean.

Pretreatment Effect on Chemical Composition of Samples. The effect of pretreatment methods on the chemical composition of samples was examined in three separate experiments on grab samples taken from Lake Anza. These grab samples were taken at different time intervals and were not identical in composition. Each of the samples was divided into four equal parts. Each part was treated with one of the four sample pretreatment methods and each chemical analysis was made in triplicate.

The results of the chemical evaluations are summarized in Table VI. Sample pretreatment apparently had no significant effect on the chemical composition of the samples because no consistent trends were observed. For instance, while the NH₃-N content was reduced by membrane filtration in two experiments, it was increased in the

TABLE VI

PRETREATMENT EFFECT ON THE CHEMICAL COMPOSITION OF SAMPLES

Chemical Analyses	Experiments	Sample Pretreatment				
		Autoclaved	Pasteurized	Whatman No. 41 Filtered	Membrane Filtered	Untreated Sample
PO ₄ -P μg/l	I	8.1 ^a	8.2	7.6	6.1	7.2
	II	6.1	6.3	6.6	5.8	4.8
	III	4.1	4.0	3.5	2.4	2.1
NH ₃ -N μg/l	I	173	143	228	232	153
	II	45.5	55.2	71.5	24	68.3
	III	64.4	88.9	70.0	16.1	49.0
NO ₃ and NO ₂ -N μg/l	I	8.6	10.9	12.5	25	10.6
	II	2.7	4.6	6.2	13.6	4.7
	III	0.9	11.5	13.2	17.3	15.3
Total-P μg/l	I	198	153	130	not det.	393
	II	105	138	122	not det.	128
	III	103	126	66.8	not det.	110
Total-N μg/l	I	147	304	216	not det.	231
	II	975	592	957	not det.	665

^a Each value represents mean of three replicate determinations.

third experiment. The same phenomena were observed for the other constituents assayed. The absence of clear trends in these analyses is probably due to the low precision of the analytical techniques at the extremely low concentrations of chemicals (all less than 1 mg/l and mostly less than 100 µg/l) encountered.

Therefore, it is conceivable that the filtration techniques, especially membrane filtration, removed substantial quantities of suspended materials (evidenced by visible green layers on filter pads after filtration), resulting in substantial losses in total nitrogen and phosphorus from solution. Unfortunately total nitrogen and phosphorus were not determined for the membrane filtered samples because of lost samples. Because only a small fraction of the total nitrogen and phosphorus occurred in dissolved inorganic form (Table VI), such losses may result in the reduction of the maximum cell production ($\bar{X}$) responses in algal assays.

Effect of Pretreatment on Algal Responses. To evaluate the effect of pretreatment on the algal responses of an assay of the treated samples, a grab sample of Lake Anza was divided into four portions. Each portion was treated with one of the pretreatment methods, i. e., pasteurization (71°C for 15 sec), autoclaving, filtration through either Whatman No. 41 or membrane (0.45-µ) filters. Thereafter four replicate batch flasks (250-ml sample in each) were prepared for each pretreatment. The flasks were inoculated with Selenastrum capricornutum and incubated under standard PAAP conditions [35] with air ventilation. Cell counts were performed daily from the 2nd to the 8th day. Terminal suspended solids were too low for accurate measurement and therefore final cell counts were made on the 14th day.

Mean Specific Growth Rate ($\hat{\mu}_b$). A comparison of the algal growth rates based on cell numbers obtained during the batch assays is given in Table VII. The highest growth rates were obtained in the autoclaved samples, followed by the pasteurized samples. The growth rates obtained with the filtration treated samples were the lowest. The growth rates in the autoclaved samples were significantly higher ($p = 0.05$) than the growth rates of either filtered treatments. The pasteurized samples did not differ significantly from the autoclaved or filtered samples; however, the variation with this treatment was the greatest. There was no significant difference between the filtered samples.

TABLE VII
MEAN ALGAL GROWTH RATES OBTAINED
AFTER SAMPLE PRETREATMENT

Treatment	Mean Maximum Specific Growth Rate ^a day ⁻¹	Coefficient of Variation ^b %
Autoclaved	0.46	26.4
Pasteurized	0.33	43.0
Filtration Whatman No. 41	0.25	24.0
Filtration Membrane 0.45-μ	0.25	29.3

^aMean growth rate of four replicate flasks; based on cell numbers.

^bCoefficient of variation = standard deviation (100)/mean.

Maximum Cell Concentration, $\hat{X}$. A comparison of the maximum cell concentration values for the different treatments is given in Table VIII. The autoclaved samples had the highest mean value but also the greatest amount of scatter (coefficient of variation = 80%). Similarly, the Whatman filtered samples had a mean of more than 100,000 cells/ml and a wide scatter ($C_v = 47\%$). These means did not differ significantly from the mean of the membrane filtered samples. However, the pasteurized sample mean (84×10^3 cells/ml), while less than those of the autoclaved and Whatman filtered samples, differed significantly (t-test with $p = 0.05$) from the mean of the membrane filtered sample. The reason for this was that there was less scatter in pasteurized replicates ($C_v = 29\%$). If the autoclaved and Whatman filtered samples had less variation they also would have differed significantly from the membrane filtered samples. The autoclaved, pasteurized, and Whatman filtered samples did not differ significantly from each other.

TABLE VIII
MEAN CELL PRODUCTION ($\bar{X}$) VALUES OBTAINED
AFTER SAMPLE PRETREATMENT

Sample Pretreatment	$\bar{X}$ Mean Maximum Cell Production (14 days) cells/ml ^a	C_v Coefficient of Variation ^b %
Autoclaved	113,000	80
Pasteurized	84,200	29
Filtration through Whatman No. 41	103,000	47
Filtration through membrane filter	33,300	14

^aMean of four replicate flasks.

^bCoefficient of variation (%) = standard deviation (100)/mean

Williams and Yeiseley [59] in a similar evaluation of sample pretreatment methods reported a 20-fold increase in maximum cell production of autoclaved samples compared to filtered (0.5- μ pore size) control. Therefore, it can be concluded that heat treatment of samples would result in a higher maximum cell production than samples treated by membrane filtration.

Summary of Sample Pretreatment Evaluation. All sample treatment methods appeared to remove indigenous algae from the samples; however Whatman filtration is likely the least effective. Autoclaving, pasteurization, and membrane filtration appear to be adequate in removing most indigenous algae.

The effects of the pretreatments on the chemical composition of the water samples seemed to indicate no great chemical changes taking place. However, filtration of samples, especially membrane filtration, which removes algae and particulate matter, must also remove some of the suspended nutrient constituents of the waters. This effect was clearly demonstrated by significantly lower algal growth rates and cell concentration values for filtered samples when compared to pasteurized samples and likely autoclaved samples.

Two problems are posed by these results, namely: 1) Should nutrients such as nitrogen and phosphorus which have become part of the biomass (algal cells) or in particulate form in a water be removed prior to assaying the water? Removal of these nutrients clearly results in lower cell production values. Thus, the capacity of the water to stimulate algal growth may be underestimated; 2) When algal growth rates are to be measured, heat treatments such as autoclaving and pasteurization will kill indigenous organisms and/or soluble particulates resulting in a release of nutrients back into the medium thereby affecting the algal assay growth rates. In the natural water these nutrients are bound and may not be available for algal growth. Hence a lower algal growth rate would be obtained and the assay of the heat treated samples will tend to overestimate the in situ algal growth rate.

Therefore, when the potential of a water to produce algal cells is determined no portion of the nutrients should be removed because these nutrients form part of the total nutrient supply. In these cases filtration techniques should not be used. When algal growth rates are to be measured the release of bound nutrients back into the sample, thereby affecting the growth rates, is to be avoided. Therefore, of the methods evaluated the membrane filtration technique appears to be the method of choice when growth rates are to be determined.

Interlaboratory Precision Tests

In accordance with the decision of the PAAP evaluation group meeting at Corvallis, Oregon in May 1970 [108], studies were conducted as part of the interlaboratory precision test for the batch culture assay. A protocol for this test was supplied by Dr. C. E. Weiss of the University of North Carolina. This protocol called for the use of a single stock culture of Selenastrum capricornutum (supplied by T. E. Maloney, Pacific Northwest Water Laboratory, Corvallis) and a single source of chemicals (supplied by C. E. Weiss, University of North Carolina). The participating laboratories were to determine the algal responses (growth rate and cell production), at specified values of pH, light intensity, and temperature, and at specific intervals, i.e., 3, 5, 7, 9, 13, 17, and 21 days. Different concentrations, i.e., 1%, 10%, 30%, and 100% of PAAP medium (Tables I and II) were used and each dilution was assayed in five replicate flasks. Standard PAAP conditions [35] were used and the use of foam plug enclosures was specified.

First Interlaboratory Precision Test. The protocol as outlined above was followed although the use of foam plugs deviated from the standard procedures [34] used in this laboratory. The results of the cell counts, suspended solids, total cell volume, and pH measurements for the first interlaboratory precision test are summarized in Table C-I, Appendix C. The temperature values did not vary outside the range

24°C ± 2°C. The mean light intensity on the shaker table was 412 ft-c (coefficient of variation = 4.0%) as measured by a Weston (Model 756) light meter. Temperature and light conditions agreed well with those specified for the standard PAAP procedures [35].

The cell counts in the different dilutions of PAAP medium indicated the virtual absence of growth in the 30%, 10%, and 1% concentrations. In the 100% concentration only four flasks showed appreciable growth. The fifth flask contained only 43,000 cells/ml at termination compared to more than 3×10^6 cell/ml for the other four flasks. It was suspected that the foam plug enclosures were toxic to Selenastrum capricornutum and only in the higher nutrient concentrations could the toxicity be overcome by the alga. This conclusion was verified by results of Scherfig et al. [76] which showed that some kinds of foam plugs are extremely toxic to Selenastrum capricornutum, and foam plug toxicity was also observed by Payne [60] and Weiss [68].

The use of foam plugs in batch culture assays cannot be recommended because of this apparent toxicity. Observations that in one batch of foam plugs some are toxic and some not, indicate that all foam plugs must be tested for toxicity before being used in algal assays [77]. This is a cumbersome procedure and it does not remove all concern about possible toxic effects. Hence the use of foam plugs should be avoided whenever possible.

Second Interlaboratory Precision Test. The interlaboratory precision test was repeated because of the foam plug toxicity experienced in the first test. Instead of foam plugs, ventilated flasks with glass beaker tops (Figure 15) were used. The same flasks used in the first precision test were used after careful washing and rinsing as specified [35]. All other conditions were similar to the first interlaboratory precision test.

The results of the second precision test are summarized in Table C-II, Appendix C. The 100% PAAP medium had four flasks which had good growth ($> 25 \times 10^6$ cells/ml). One flask showed very little growth (only 0.19×10^6 cells/ml). It was evident that growth inhibition occurred in this flask. This was the same flask (A4) which showed toxicity in the first precision test. Evidently residual toxicity, which was not removed even by the cleaning procedure, remained in this flask and exhibited its growth inhibition during the second test. This flask was discarded and the results for this flask were ignored in all further comparisons.

For the 30% PAAP concentration (B series of flasks, Table C-II, Appendix C) none of the flasks showed residual toxicity from the first precision test. For the 10% PAAP medium (C series), however, three flasks showed residual toxicity effects (C1, C3, and C4). The two other flasks had terminal cell numbers in excess of 1.7×10^6 cells/ml.

The results of the flasks showing residual toxicity were ignored in all further comparisons. The 1% PAAP medium (series D) showed appreciably more growth than in the first precision test (20×10^3 cells/ml versus 2.0×10^3 cells/ml). These levels were still too low for accurate suspended solids measurements and because the nutrient levels in 1% PAAP medium are on the borderline of the sensitivity of batch assays, the results of the 1% PAAP medium assays were ignored in all further comparisons.

Maximum Cell Concentration, $\hat{X}$.

Relation with Concentration of Nutrients. One of the basic requirements of an algal assay is that there should be a proportional increase in the maximum cell concentration, $\hat{X}$, with an increase in the concentration of the maximum cell concentration limiting nutrient. Only then can a higher $\hat{X}$ be interpreted as indicating a higher concentration of the maximum cell-limiting nutrient.

$\hat{X}$ Based on Suspended Solids. The response of the $\hat{X}$ based on suspended solids for the 10, 30, and 100% concentrations of PAAP medium was significantly correlated ($p = 0.99$) with the concentration of PAAP medium in accordance with the following linear equation which is shown in Figure 18:

$$\hat{X} \text{ (suspended solids, mg/l)} = 50.43 + 3.31 (\% \text{ PAAP}). \quad (49)$$

However, according to theory, the intercept for such a relationship should be zero because no growth should occur in the absence of nutrients. Nutrient carryover with the inoculum might result in the presence of a small positive intercept but certainly it should not be as high as 50 mg/l. The intercept of the regression is far in excess of what is expected. One reason for this anomaly might be that something else other than nutrients in the medium was limiting the maximum growth in the 100% PAAP medium cultures. This would result in a depressed regression line with a large intercept.

To test the above hypothesis, a regression equation was computed omitting the 100% PAAP values and using only the values for the 10% and 30% PAAP medium cultures. The following relationship between $\hat{X}$ (suspended solids) and concentration of PAAP medium was obtained.

$$\hat{X} \text{ (suspended solids, mg/l)} = 0.76 + 5.45 (\% \text{ PAAP}). \quad (50)$$

This relationship was highly significantly correlated ($p = 0.99$, $n = 7$). It is obvious that the inclusion of the 100% PAAP values depressed the regression line. It appears that exclusion of these values in Equation 50 best represents the response of *Selenastrum capricornutum* to PAAP medium. It should be noted that both Equations 49 and 50 have higher regression coefficients than those for the relationship reported by Porcella et al. [34]. That relationship also had a high positive intercept (30.69) which also indicates that at the higher concentrations of the PAAP medium something other than nutrients was limiting growth.

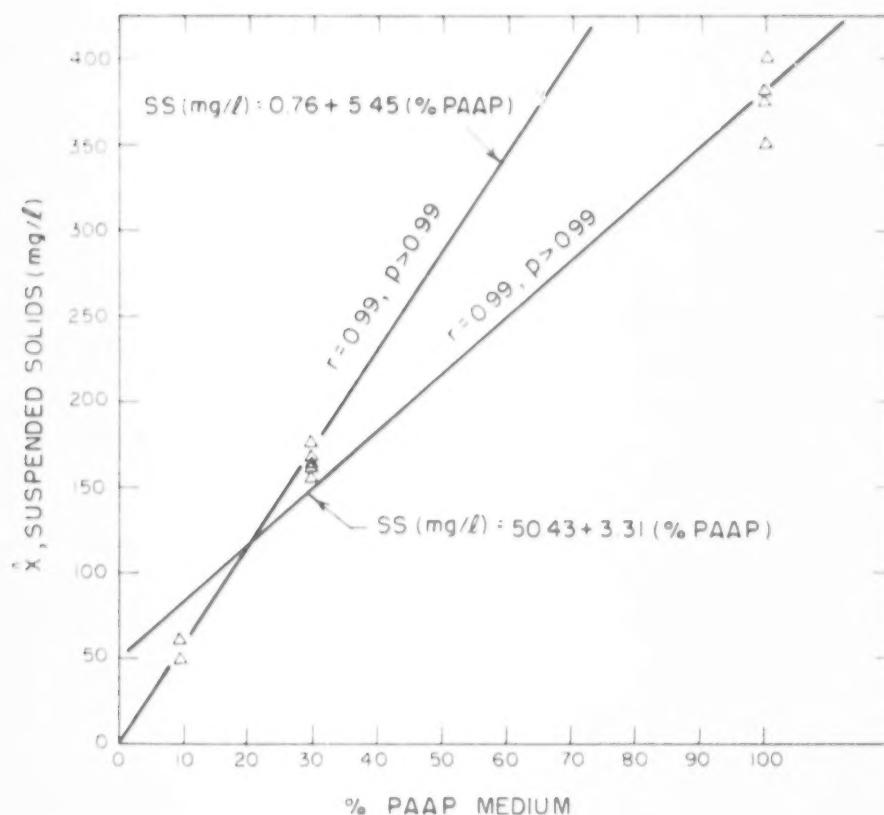


FIGURE 18. RELATIONSHIP OF *Selenastrum capricornutum* RESPONSE (SUSPENDED SOLIDS) TO CONCENTRATION OF PAAP MEDIUM

$\hat{X}$ Based on Cell Numbers. A highly significant ($p = 0.99$) correlation ($r = 0.98$) was observed between $\hat{X}$ based on cell numbers and concentration of PAAP medium (10%, 30%, and

100%). The regression equation which expresses this relationship is shown on Figure 19 and is as follows:

$$\hat{X} \text{ (number of cells, } 10^6/\text{ml)} = 0.319 (\% \text{ PAAP}) - 1.13. \quad (51)$$

If the 100% PAAP cultures are omitted the regression becomes:

$$\hat{X} \text{ (number of cells, } 10^6/\text{ml)} = 0.314 (\% \text{ PAAP}) - 1.0. \quad (52)$$

These two equations are virtually identical and based upon cell members the response to the 100% PAAP cultures were proportional to those of the 10% and 30% cultures. Both regression equations had significantly higher regression coefficients (0.319 and 0.314 versus 0.092) than those for the relationship reported by Porcella *et al.* [34]. The difference cannot be explained except that two different strains of *Selenastrum capricornutum* were used and it may reflect some of the unexplained anomalies associated with the bottle assay.

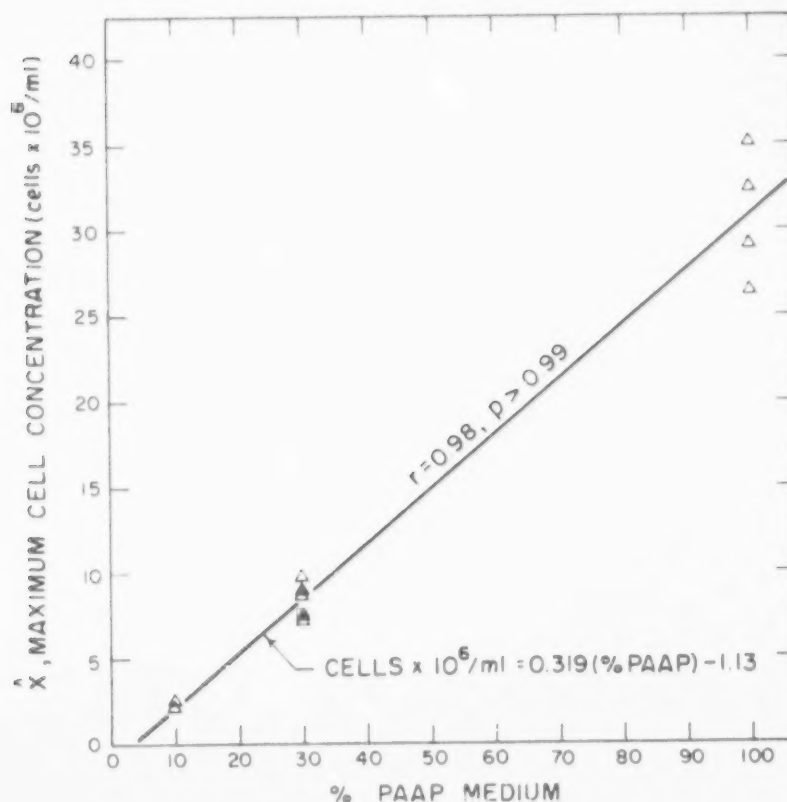


FIGURE 19. RELATIONSHIP OF *Selenastrum capricornutum* RESPONSE (CELL NUMBERS) TO CONCENTRATION OF PAAP MEDIUM

With $\hat{X}$ based on cell numbers there was no depression of the regression line with a resulting increase in the intercept as for $\hat{X}$ based on suspended solids. From the $\hat{X}$ based on cell numbers it would appear that nothing but the nutrients in the culture media limited the maximum growth of the alga. This is in direct contrast to the results of $\hat{X}$ based on suspended solids, which again reflects the variabilities associated with bottle assays.

$\hat{X}$ Based on Total Cell Volume. A highly significant ($p = 0.99$) correlation coefficient ($r = 0.97$, $n = 11$) was observed between $\hat{X}$ based on total cell volume and concentration of PAAP medium (10%, 30%, and 100% PAAP). The regression equation which expresses this relationship is shown on Figure 20 and is as follows:

$$\hat{X} \text{ (total cell volume, } \mu^3/\text{ml)} = 19.34 (\% \text{ PAAP}) - 24.2, \quad (53)$$

Again if the 100% PAAP culture values are not taken into account the relationship is:

$$\hat{X} \text{ (total cell volume, } \mu^3/\text{ml)} = 19.93 (\% \text{ PAAP}) - 38.0, \quad (54)$$

These two equations are very similar and the response of the 100% cultures were nearly proportional to those of the 10% and 30% cultures. The inclusion of the 100% culture values did not result in a depression of the regression line with a resultant increase in the intercept. In this regard these values agree with those of $\hat{X}$ based on cell numbers and not with those of $\hat{X}$ based on suspended solids. However, a negative intercept of 0% PAAP (-24 and -38) is difficult to explain on a theoretical basis.

Precision of $\hat{X}$ Estimates. The precision of the analyses for $\hat{X}$ for the 100% and 30% PAAP cultures was estimated and is summarized in Table IX.

The suspended solids measurements had the lowest coefficient of variation (approximately 5%) while the cell numbers and total cell volume estimates had coefficients of variation of approximately 13% and 11%, respectively. These values are in the same range (approximately 10% to 15%) as the values reported by Porcella et al. [34]. Therefore, the coefficient of variation of batch algal assays appears to be in the order of 15%: a precision which seems to be acceptable in a biological assay technique.

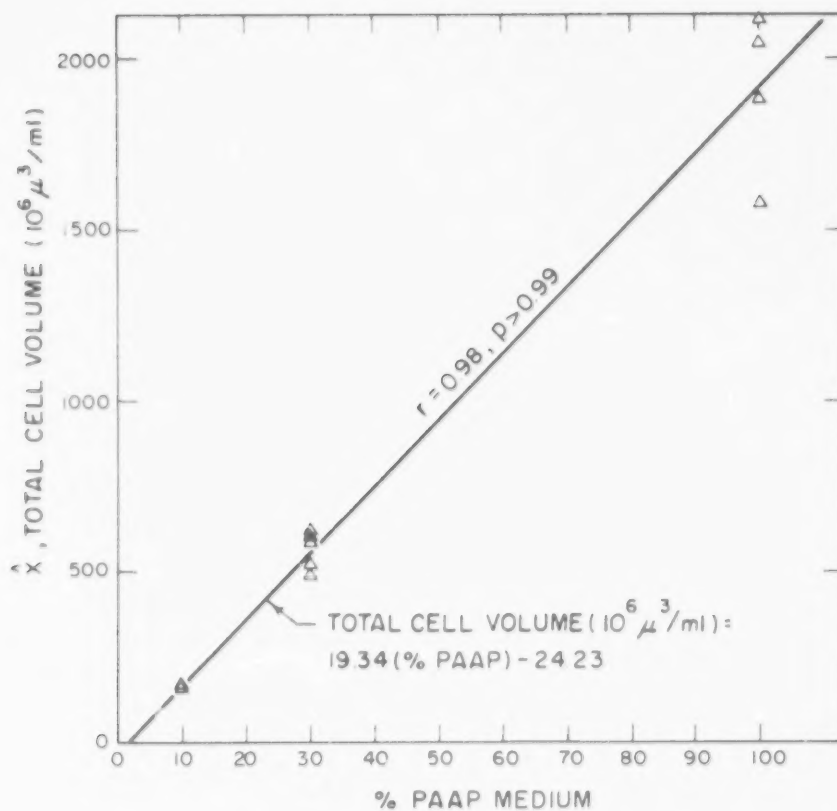


FIGURE 20. RELATIONSHIP OF *Selenastrum capricornutum* RESPONSE (TOTAL CELL VOLUME) TO CONCENTRATION OF PAAP MEDIUM

TABLE IX
MEAN VALUES AND PRECISION ESTIMATES FOR $\hat{X}$
BASED ON DIFFERENT BIOMASS PARAMETERS

Maximum Cell Concentration ($\hat{X}$) Based on	100% PAAP		30% PAAP		10% PAAP ^b Mean
	Mean	$C_v(\%)^a$	Mean	$C_v(\%)^a$	
Suspended solids, mg/l	377	5.5	164.0	4.8	55.3
Cell numbers, 10^6 /ml	30.8	12.6	8.4	13.2	2.1
Total cell volume, $10^6 \mu^3$ /ml	1910.0	12.7	560.0	10.1	161.0

^a $C_v(\%)$ = coefficient of variation = 100 (standard deviation)/mean.

^bCoefficient of variation could not be determined because of insufficient number of replicates.

Discussion and Summary of Maximum Cell Concentration ($\hat{X}$) Response The precision of the $\hat{X}$ response based on any of the biomass parameters, i. e., suspended solids, cell numbers, or total cell volumes, was acceptable ($C_v \leq 15\%$) for a biological assay technique. However, there appeared to be a difference in the type of response from the different biomass parameters, i. e., a growth limitation by something other than the culture nutrients was observed with $\hat{X}$ based on suspended solids but not with $\hat{X}$ based on either cell numbers or total cell volumes.

The difference in behavior can be explained if the mass per cell decreased with increased nutrient concentrations in the culture media. An examination of the mean cell volumes showed that the mean cell volume of *Selenastrum capricornutum* in 100% PAAP was $62 \mu^3$ /cell ($C_v = 3.3\%$); this increased to $66.9 \mu^3$ /cell ($C_v = 7.1\%$) for 30% PAAP and to $79.5 \mu^3$ /cell ($C_v = 13.3\%$) for 10% PAAP, respectively. The regression line, i. e.,

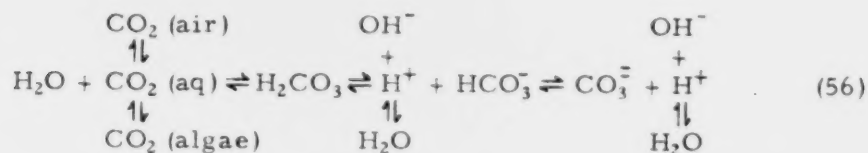
$$\text{Mean cell volume } (\mu^3/\text{cell}) = 74.3 - 0.13 (\% \text{ PAAP}) \quad (55)$$

which describes this relationship had a significant correlation coefficient ($r = 0.55$, $n = 22$, $p = 0.99$). If the density of cells

grown in different concentrations of PAAP medium is constant, then the mass/cell decreases as the nutrient concentration in the culture medium increases. Therefore a difference in the regression between $\hat{X}$ based on suspended solids and on cell numbers should be observed. Variations in the cell size of Selenastrum capricornutum under different culturing conditions (with or without carbon dioxide addition) have been reported also by Dixon and Scherfig [55].

The significant relationship between $\hat{X}$ based on total cell volumes and concentration of PAAP medium indicates that the effect of cell size variation was not sufficient to account for the difference in $\hat{X}$ response. If it were sufficient then the $\hat{X}$ based on total cell volumes should have shown the same relation as $\hat{X}$ based on suspended solids. However, the higher precision of the suspended solids measurements suggests that the depressed values for 100% PAAP were not artifacts but were due to a growth limitation by something other than the nutrients in the culture media. It is possible that either carbon dioxide or light may have limited the growth in the higher nutrient concentration samples. Because it has been shown that the use of 800 ft-c light intensities showed no significant differences from the use of 400 ft-c intensities [58] it is doubtful if light limitation was experienced. Therefore, carbon limitation was probably responsible for the anomaly.

Carbon dioxide is a part of the carbon dioxide-bicarbonate-carbonate buffer system of waters, i. e.,



In the case where algae are utilizing CO_2 at a higher rate than at which it can be replenished from the air, bicarbonates and carbonates will be converted to $\text{CO}_2 (\text{aq})$ resulting in the production of hydroxyl ions and a concomitant rise in pH. Examination of the pH values of the cultures (Table C-II, Appendix C) shows that the pH in the 100% cultures (A series) was 8.6 or above on the 9th day. The pH in the culture which had little growth because of toxicity (A4) reached only 8.4 at maximum. The 10% and 30% PAAP cultures reached maximum pH values of 8.0 and 8.5, respectively. Therefore, the greater the growth in the cultures, the higher the maximum pH observed. This also suggests that carbon limitation could have occurred in the 100% cultures.

Carbon limitation during the assaying of high nutrient concentration samples is a distinct possibility. If carbon is not the nutrient of interest, extreme care must be exercised to prevent carbon limitation. The maintenance of culture pH values between 7.0 and 8.3 through the use of correct surface/volume ratios in flasks or through the use of ventilation systems has been recommended [77]. However, the above results indicate that even with a ventilation system in operation, pH values in high-nutrient concentration samples can increase sufficiently to experience carbon limitation. Increased air flow rates are necessary to prevent such limitations, and it appears that the use of flasks with specified surface/volume ratios is no guarantee that carbon limitation will not occur in high nutrient concentration samples. In such samples, e. g., undiluted secondary effluents, the pH of the cultures must be monitored frequently and be kept in the range 7.0 to 8.3.

One point of caution should be noted in interpreting the foregoing data as to the adequacy of the batch assay using the maximum cell concentration, $\hat{X}$, as the response parameter with respect to nutrient concentrations (% PAAP medium). The lowest concentration of nutrients used (10% PAAP) resulted in a maximum cell concentration, $\hat{X}$, of about 55 mg/l of cells at the end of 21 days incubation. This concentration of cells is much higher than normally encountered in highly eutrophied waters with the possible exception of a severe bloom condition. As this is near the lowest level of sensitivity of the batch assay and all other nutrient concentrations assayed were higher than 10% PAAP $\sim 62 \mu\text{g P/l}$, it is questionable that these correlations are very indicative of the accuracy of the batch assay for assaying natural waters with moderately low concentrations of nutrients (i. e., $< 60 \mu\text{g P/l}$, $< 140 \mu\text{g N/l}$). As was noted earlier, the results of the batch assays on the 1% PAAP medium ($6 \mu\text{g P/l}$ and $14 \mu\text{g N/l}$) were omitted from the regression analyses because of the lack of sensitivity at this level of nutrients. It may be that the most important range of nutrient concentration that need to be assayed are in the range equivalent to 1% to 10% PAAP medium – and this is the range of concentration where the batch assay has yet to be shown to be interpretative or applicable.

Maximum Specific Growth Rates, $\hat{\mu}_b$. The mean maximum specific growth rate (based on cell counts) of *Selenastrum capricornutum* in the 100% PAAP medium was 1.95 day^{-1} with a coefficient of variation of 14.6%. For the 30% PAAP it was 2.06 day^{-1} with a coefficient of variation of 15.8%. The value for $\hat{\mu}_b$ for the 10% PAAP was 1.53 day^{-1} ; and due to the lack of sufficient number of replicates an estimate of the variance could not be made. However, the results seem to indicate that there was no difference in the growth rates of the 100% and 30% PAAP media and that a somewhat

lower growth rate was obtained in the 10% samples. Total cell volume estimates could not be used to determine $\hat{\mu}_b$ because the instrument for determining mean cell volumes was under repair at that time.

The absence of large or significant differences between growth rates ($\hat{\mu}_b$) obtained in different strength samples should not be interpreted as growth rate being an inferior response measurement. Differences in the growth rates for an organism is dependent on the ability of the organism to utilize a rate-limiting nutrient efficiently at very low concentrations. This ability is reflected by the half saturation constant, K_s , of the organism for a specific nutrient, i. e., the concentration of the nutrient at which the organism will attain half the maximum specific growth rate ($\hat{\mu}$). In the case of PAAP medium it appears likely that phosphorus is the primary limiting nutrient. Porcella *et al.* [34] reported that the K_s value for phosphorus for *Selenastrum capricornutum* was less than 25 $\mu\text{g P/l}$ while later in this report evidence is presented that the K_s for phosphorus is as low as 3-6.0 $\mu\text{g P/l}$. Therefore, in PAAP medium, with phosphorus being the limiting nutrient, differences in growth rates for *Selenastrum capricornutum* cannot be observed unless the phosphorus concentration is reduced to very low levels, i. e., less than about 2 to 3 times the K_s value or about 6 to 15 $\mu\text{g/l}$ as P. This is markedly less than the P concentration in 10% PAAP (62 $\mu\text{g/l}$) so that no differences in growth rates would be expected except for other limiting factors between the 10%, 30%, and 100% PAAP concentrations.

Natural Water Assays

Batch algal assays of Clear Lake and Sacramento River water samples were undertaken to determine: 1) the linearity of response and the precision of maximum cell concentration ($\hat{X}$) in the water samples, 2) the effect of dilution on the growth rate ($\hat{\mu}_b$), 3) the effect and linearity of response of the addition of secondary effluent addition to samples, and 4) the identification of the growth-limiting nutrient in the samples.

Water samples were obtained and were pretreated either by pasteurization or autoclaving. Thereafter the water samples were divided into the necessary aliquots. Linearity of response of $\hat{X}$ and also the presence of toxic materials were investigated by dilution of Clear Lake or Sacramento River water with distilled deionized water to concentrations of 33.3% and 66.7% (V/V). These dilutions as well as 100% Clear Lake water were assayed in triplicate. The effect of dilution on the algal growth rate was also investigated in these dilutions. Addition of secondary effluent in concentrations of 10%, 20%, and 30% (V/V) to undiluted Clear Lake or Sacramento River water was used to determine the effect of addition of secondary effluent. Triplicate

assays were made on each concentration of secondary effluent, and the effect of secondary effluent addition on the algal growth rate was also followed. Enrichment (spiking) procedures, aimed primarily at nitrogen, phosphorus, and iron were used to determine the growth-limiting nutrient. Micronutrients and macronutrients were respectively used as combined spikes. The spiking procedure is summarized in Table X.

TABLE X
EXPERIMENTAL PROCEDURE FOR IDENTIFICATION
OF GROWTH-LIMITING NUTRIENT

Enrichment (Spike) of Clear Lake Water	Replicate Flasks
None	3
N ^a	2
P ^b	2
Fe ^c	2
N, P	2
N, Fe	2
P, Fe	2
N, P, Fe	2
N, P, Fe, Micronutrients ^d	2
30% PAAP medium ^e	2
Secondary effluent ^f	3
Control ^g	1

^aSpiked with NaNO₃, final concentration after spike 2.5 mg N/l + [N] of sample.

^bSpiked with KH₂PO₄, final concentration after spike 200 µg P/l + [P] of sample

^cSpiked with FeCl₃, final concentration after spike 20 µg Fe/l + [Fe] of sample

^dMicronutrient spike identical in composition and concentration to that of 30% PAAP medium.

^eFinal concentration of nutrients after spike equal to 30% PAAP medium.

^fSecondary effluent from pilot plant using activated sludge procedures.

^gControl was 30% PAAP medium.

All spike concentrations were selected to increase $\hat{X}$ with at least 50 mg/l (suspended solids) based upon the yield coefficients presented by Porcella *et al.* [34].

Clear Lake Studies. During the Clear Lake assays control flasks of 30% PAAP medium produced a $\hat{X}$ of suspended solids of 148.3 mg/l ($C_V = 4.6\%$) and of cells $\times 10^6/\text{ml}$ of 3.164 ($C_V = 9.2\%$). In addition, in Clear Lake water supplemented with 9% PAAP medium an $\hat{X}$ of suspended solids of 102.5 mg/l ($C_V = 8.5\%$) and of cells $\times 10^6/\text{ml}$ of 2.24 ($C_V = 4.9\%$) were produced. Therefore, the inoculum appeared to be responsive to nutrients and growth occurred as expected.

Maximum Cell Concentration ($\hat{X}$) and Toxicity. Maximum cell concentration ($\hat{X}$) based on suspended solids, cell counts, and total cell volume were determined. The growth curves for all assays are presented in Table C-III, Appendix C.

Undiluted Clear Lake water produced 39.1 mg/l suspended solids ($C_V = 6.3\%$). Clear Lake water diluted to 66.7% produced 27.4 mg/l suspended solids, but the Clear Lake water diluted to 33.3% produced nearly the same (26.6 mg/l). This higher-than-expected value in the 33.3% samples cannot be explained. The best fit relationship between suspended solids and the 66.7% and 100% cell concentrations is shown in Figure 21.

Undiluted Clear Lake water produced 0.573×10^6 *Selenastrum* cells/ml ($C_V = 6.7\%$). Correspondingly, the 66.7% dilution produced 0.452×10^6 cells/ml. There was a highly significant relationship ($r = 0.97$, $n = 9$) between the dilution of Clear Lake water and the $\hat{X}$ (cell counts) as shown in Figure 22. Similarly, a highly significant ($p \geq 0.99$) relation was observed between $\hat{X}$ based on total cell volume and the dilution of Clear Lake water ($r = 0.9$, $n = 9$) as shown in Figure 23.

Dilution of Clear Lake water appeared to yield a proportional decrease in the $\hat{X}$ based on suspended solids, cell counts, or total cell volume (Figures 21, 22, and 23). No evidence was obtained of the presence of toxicants as observed by Porcella *et al.* [34]. The toxicity observed in that study must have developed upon the prolonged storing of samples, even in the dark at 2°–4°C. The prolonged storage of water samples before assaying should be avoided.

No ready explanation is available for the lack of linearity of response between $\hat{X}$ and percent sample when $\hat{X}$ was based upon the cell concentration parameter, suspended solids. Neglecting the values for the 33% samples, the ordinate corresponding to 0% was still about 15% of the response observed for the 66% sample.

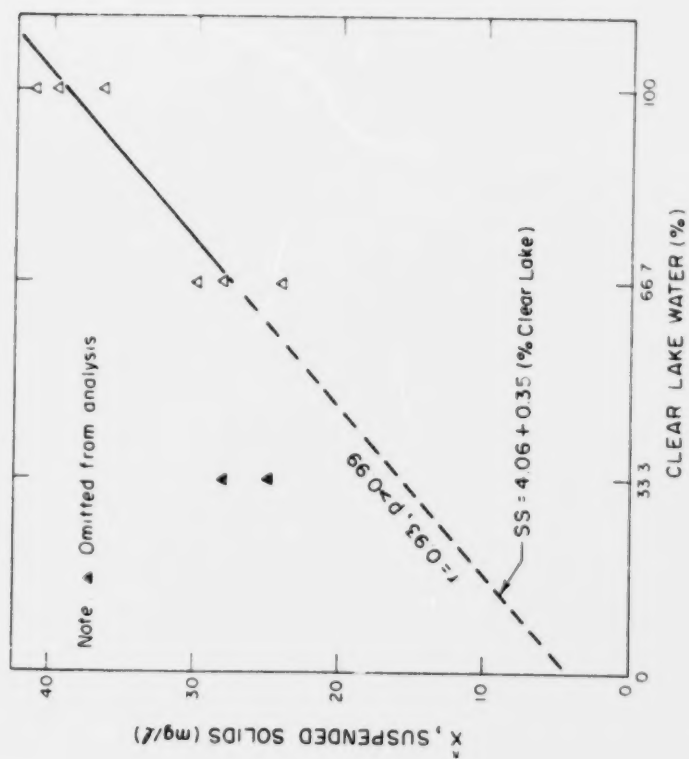


FIGURE 21. RELATIONSHIP BETWEEN DILUTION OF CLEAR LAKE WATER AND $\bar{X}$ BASED ON SUSPENDED SOLIDS

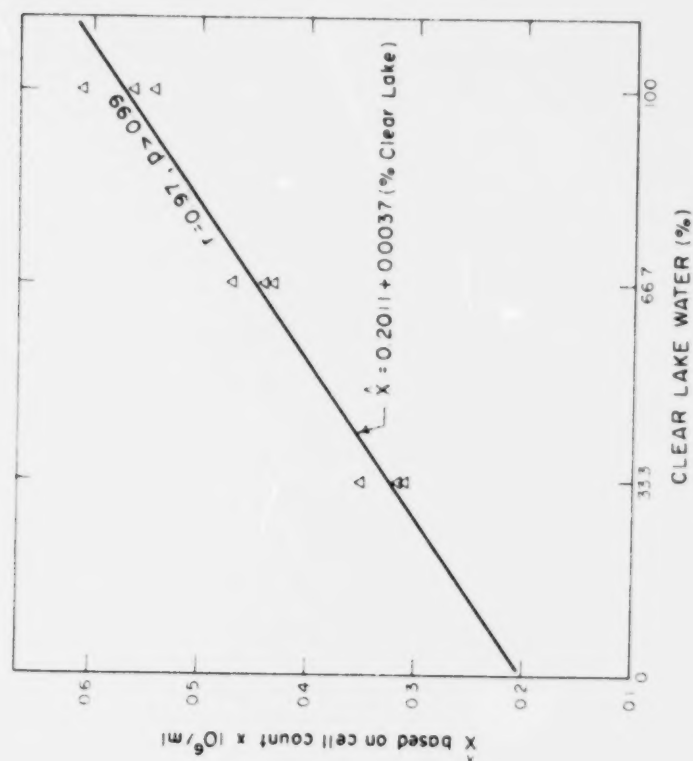


FIGURE 22. RELATIONSHIP BETWEEN DILUTION OF CLEAR LAKE WATER AND $\bar{X}$ BASED ON CELL NUMBERS

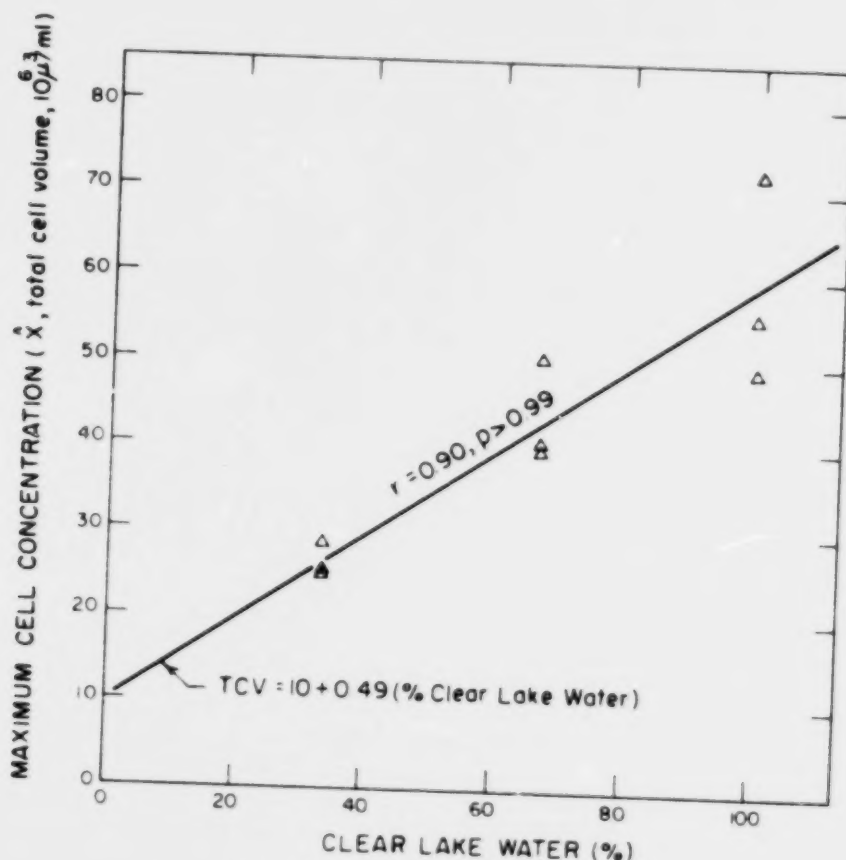


FIGURE 23. RELATIONSHIP BETWEEN DILUTION OF CLEAR LAKE WATER AND $\hat{X}$ BASED ON TOTAL CELL VOLUME

Yet, the growth response was linear with concentration for $\hat{X}$ based upon cell numbers and cell volume. However, in both cases the 0% sample growth response (ordinate) varied from about 15% to as high as 35% of the response for the 100% sample. No explanation for this anomaly is available.

Precision of Biomass Estimates. The mean values and the precision of the biomass estimates are summarized in Table XI. The coefficient of variation of the different maximum cell concentration estimates were all less than 11%. The precision of all biomass measurements were adequate although only 3 replicates were used for each of the dilutions. Consequently, the use of 3 replicates per sample during assays is recommended.

TABLE XI
MEANS AND PRECISION OF MAXIMUM CELL CONCENTRATION
ESTIMATES IN CLEAR LAKE WATER

Maximum Cell Production Based On:	Clear Lake Water					
	33.3% ^a		66.7% ^c		100% ^d	
	Mean	C _v ^b	Mean	C _v	Mean	C _v
Suspended solids, mg/l	26.6	c	27.4	11.0 ^d	39.1	6.3
Cell numbers, 10 ⁶ /ml	0.324	8.4	0.452	4.8	0.573	6.7
Total cell volume, 10 ⁶ μ ³ /ml	26.0	7.9	37.2	5.1	50.9	8.4

^a Diluted with distilled deionized water.

^b Coefficient of variation = standard deviation (100)/mean.

^c Lost sample during assay prevented C_v calculation.

^d No explanation is available for the C_v of $\hat{X}$ (suspended solids) being higher than those for $\hat{X}$ (cell numbers) or $\hat{X}$ (total cell volume).

Maximum Specific Growth Rate, Batch ($\hat{\mu}_b$) in Clear Lake Water.

The effect of dilution on the growth rate is shown in Figure 24 and Table XII. Dilution of Clear Lake water reduced the algal growth rate as measured by either cell count or total cell volume measurements. However, the $\hat{\mu}_b$ estimates based on total cell volume measurements were always less than the $\hat{\mu}_b$ estimates based on cell counts. The reason for this discrepancy is not apparent.

Dilution of Clear Lake water did not reduce the growth rate linearly, e. g., one-third dilution reduced the growth rate only 8.3% based on cell count and 21.1% based on total cell volume. Two-thirds dilution reduced the algal growth rate only 24.2% based on cell counts and 31.1% based on total cell volume. This would be expected from a situation where the nutrient concentration is in the zero order part of a first order-zero order function between growth rate and limiting-nutrient concentration (e. g., M-M, M equation). Dilution would not be expected to reduce the growth rate linearly. Clear Lake water must be diluted appreciably (> 66%) before the algal growth rate is appreciably (> 30%) reduced.

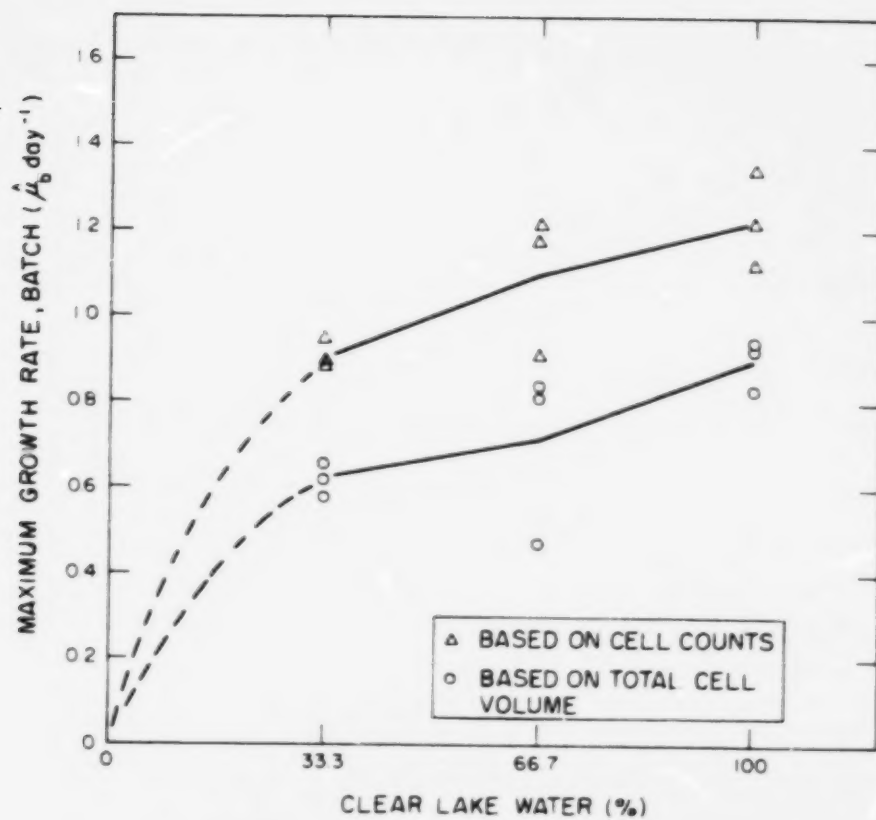


FIGURE 24. MAXIMUM BATCH GROWTH RATES ($\hat{\mu}_b$) IN CLEAR LAKE WATER

TABLE XII
MEANS AND PRECISION OF ALGAL GROWTH RATE
ESTIMATES FOR CLEAR LAKE WATER

Sample	Maximum Specific Growth Rate, $\hat{\mu}_b$, day ⁻¹ Based On			
	Cell Counts (10 ⁶ cells/ml)		Total Cell Volume (10 ⁶ μ^3 /ml)	
	Mean	C _v % ^a	Mean	C _v % ^a
100% CL ^b	1.22	9.0	0.90	6.5
67% CL	1.10	15.0	0.71	29.1
33% CL	0.91	4.1	0.62	6.5
CL + 9% PAAP ^c	2.24	5.4	1.91	3.9
30% PAAP	3.16	9.0	2.82	4.8
CL + 10% SE ^d	3.09	2.4	2.66	4.9
CL + 20% SE	3.05	6.1	2.50	15.3
CL + 30% SE	2.82	4.8	2.38	6.8

^aC_v% = Coefficient of variation = standard deviation (100)/ $\bar{X}$.

^bCL = Clear Lake water.

^cPAAP = PAAP medium as given in Tables I and II.

^dSE = Secondary effluent.

The coefficients of variation of the $\hat{\mu}_b$ estimates were less than 15% for all cases except one, which is only slightly higher than that observed for $\bar{X}$.

Utilization of Inorganic Nutrients During Assays. The chemical composition of inorganic nitrogen and phosphorus compounds in the Clear Lake samples during the assays is summarized in Table XIII. All values are the means of triplicate analyses except for the nitrate and nitrite nitrogen (NO₃-N and NO₂-N) measurements in which the 3 aliquots were pooled before analysis. Nitrogen was present in the undiluted (100%) Clear Lake sample

TABLE XIII
MEANS OF INORGANIC NITROGEN AND PHOSPHORUS CONCENTRATIONS IN CLEAR LAKE WATER

Clear Lake Concentration	Initial Concentration, S ₀ μg/l				Terminal Concentration, S _t (14 days, μg/l)			
	NH ₃ -N		NO ₃ + NO ₂ -N		NO ₃ + NO ₂ -N		PO ₄ -P	
	Mean	C _v ^a	Mean	C _v	Mean	C _v	Mean	C _v
100%	109.9	2.9	80.7	387	8.4	65.1	6.0	5.0
66.7%	59.9	2.0	52.2	229	4.2	13.7	4.2	12.1
33.3%	37.1	3.3	25.5	127.5	2.5	46.1	2.1	14.3

^aCoefficient of variation = 100 (standard deviation)/mean.

at a concentration of approximately 190 $\mu\text{g N}/\ell$. Ammonia nitrogen ($\text{NH}_3\text{-N}$) contributed 110 $\mu\text{g}/\ell$ and NO_3 and $\text{NO}_2\text{-N}$ contributed 80 $\mu\text{g}/\ell$. Phosphate phosphorus ($\text{PO}_4\text{-P}$) was 387 $\mu\text{g}/\ell$. The initial inorganic N/P ratio of 0.49 suggested that nitrogen rather than phosphorus would be the growth-limiting nutrient; however, some organic nitrogen contained in particulate material in the water samples could have become available upon hydrolysis during the assays.

The accuracy of dilution of the Clear Lake water was illustrated by the highly significant ($p = 0.99$) correlation coefficients between either initial $\text{NH}_3\text{-N}$ concentrations or $\text{PO}_4\text{-P}$ concentrations and the dilution concentration of the Clear Lake water. A correlation coefficient of 0.97 ($n = 9$) was obtained for the relationship:

$$\text{NH}_3\text{-N } (\mu\text{g}/\ell) = 3.91 + 1.09 (\% \text{ Clear Lake water}), \quad (57)$$

and a correlation coefficient of 0.99 ($n = 9$) was obtained for the relationship:

$$\text{PO}_4\text{-P } (\mu\text{g}/\ell) = 3.89 (\% \text{ Clear Lake water}) - 11.5 \quad (58)$$

During the assays almost 90% of the NO_3 and $\text{NO}_2\text{-N}$ was utilized (removed from solution) in all dilutions of Clear Lake water. Terminal nitrogen concentrations were therefore less than 10 $\mu\text{g N}/\ell$ (Table XIII). More than 98% of the $\text{PO}_4\text{-P}$ was taken up in all dilutions resulting in terminal phosphorus concentrations of 6 $\mu\text{g P}/\ell$ or less. Unfortunately the terminal $\text{NH}_3\text{-N}$ concentration was not determined, but it is expected that most of the $\text{NH}_3\text{-N}$ was also utilized because $\text{NH}_3\text{-N}$ is a ready nitrogen source for most algae. Very substantial reductions in the concentrations of inorganic nitrogen and phosphorus compounds was experienced during the assays.

Yield Coefficients for Nitrogen and Phosphorus. The yield coefficients (mass of cells produced/mass of nutrient removed) for $\text{NO}_3\text{-N}$ ($Y_{\text{NO}_3\text{-N}}$) were respectively 540, 571, and 1150 in the 100%, 66.7%, and 33.3% dilutions of Clear Lake water. If it is assumed that all the $\text{NH}_3\text{-N}$ in the samples was also consumed for cell production, the Y_N values would be 214, 254, and 443, respectively, for the 100%, 66.7%, and 33.3% cultures. These values are approximately an order of magnitude higher than the Y_N values reported by Porcella *et al.* [34]. The inverse of the yield coefficients, i.e., mass of nutrient/mass of cells, gives an indication of the concentration of a specific nutrient in the cells. Inversion of the Y_N values for the different dilutions suggests that the cells contained respectively only 0.45%, 0.39%, and 0.23% nitrogen on a dry weight basis. Compared to the values

of 3% to 5% reported by Porcella *et al.* [34] and 1.77 to 9.43% nitrogen reported [110] for various algae. The yield values of the present study appear to be extremely low. The only explanation for this is that organic nitrogen present in the particulate matter in the water samples was hydrolyzed and used for cell production during the assays. However, it should be noted that according to Holm-Hansen *et al.* [111] the concentration of nitrogen in nitrogen-starved cells can be very low.

Because of the relatively high mass of cells produced per unit of inorganic nitrogen and because of the possibility of organic nitrogen being hydrolyzed and made available for cell synthesis, it cannot be established definitely whether or not nitrogen was the limiting nutrient in the Clear Lake samples. However based upon the available information, it appears that N was limiting.

The Y_p values observed, i. e., 103, 122, and 212, for the 100%, 66.7%, and 33.3% samples respectively, were lower than those of Porcella *et al.* [34], i. e., 800 to 1000, for phosphorus-limiting conditions. Inversion of the Y_p values suggests that the cells contained respectively 0.97%, 0.82%, and 0.47% phosphorus on a dry weight basis. These values are in the range of 0.52% to 1.89% phosphorus (dry weight) for various algae, compiled by McGauhey *et al.* [31]. However, they are much greater than the values of approximately 0.1% observed [34] when phosphorus was the growth-limiting nutrient. The data indicate that phosphorus did not limit algal growth in the Clear Lake samples.

The use of yield coefficients to aid in identifying growth-limiting nutrients in batch assays appears to be limited. This is due to large variations in the yield coefficients due to excess uptake such as observed by Porcella *et al.* [34] and in this study coupled with the possible interaction of organic and inorganic forms of nutrients such as nitrogen.

Secondary Effluent Addition to Clear Lake Water.

Maximum Cell Concentration ($\hat{X}$). The $\hat{X}$ based on three different biomass parameters were increased by the addition of secondary effluent to Clear Lake water. The growth curves are summarized in Table C-IV, Appendix C.

In all instances a highly significant ($p \geq 0.99$) linear relationship was observed between the amount of secondary effluent added and the resulting $\hat{X}$. These relationships are summarized in Figures 25, 26, and 27. The following relationships were obtained.

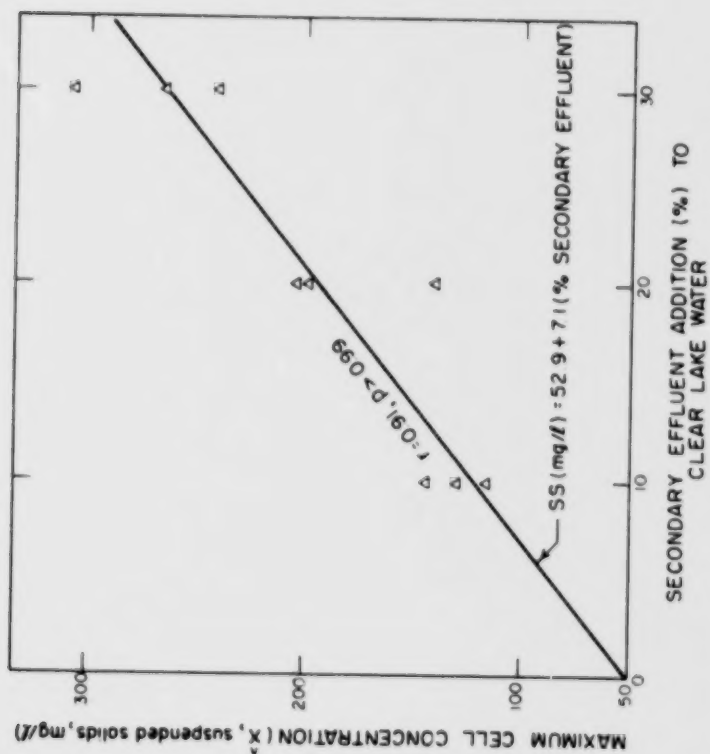


FIGURE 25. EFFECT OF SECONDARY EFFLUENT ADDITION ON $\bar{X}$ BASED ON SUSPENDED SOLIDS OF CLEAR LAKE WATER

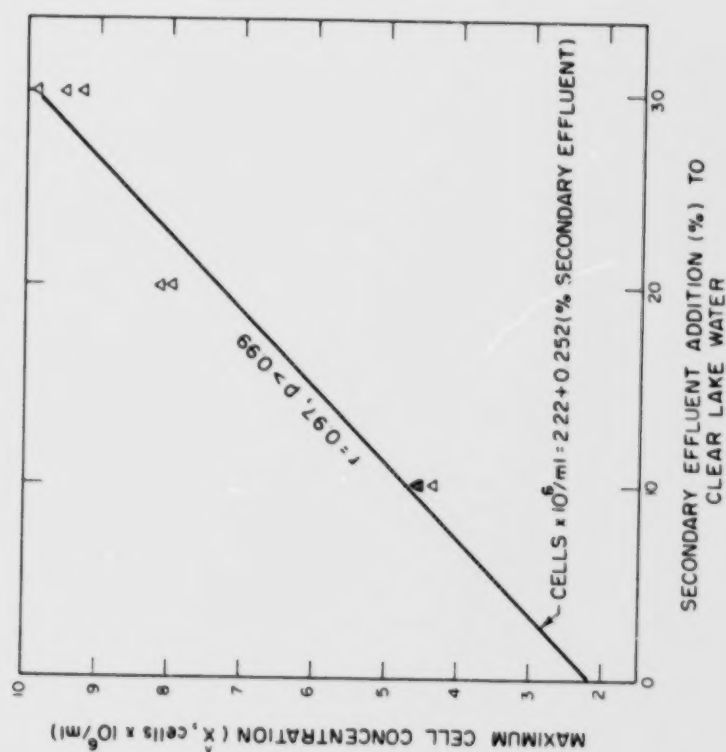


FIGURE 26. EFFECT OF SECONDARY EFFLUENT ADDITION ON $\bar{X}$ BASED ON CELL NUMBERS OF CLEAR LAKE WATER

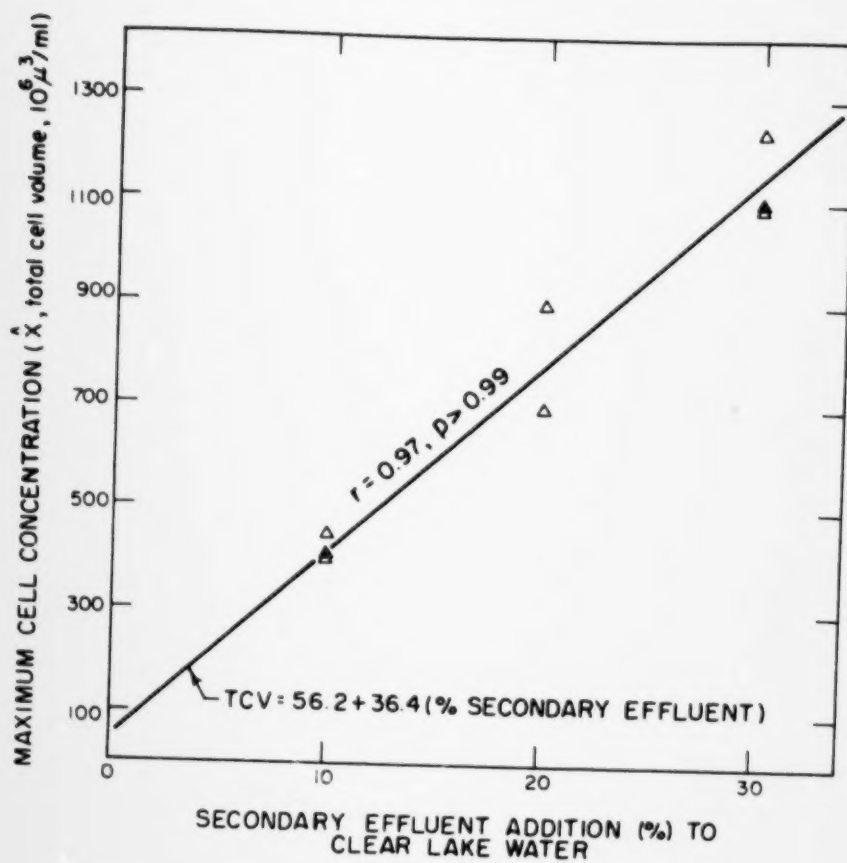


FIGURE 27. EFFECT OF SECONDARY EFFLUENT ADDITION ON $\hat{X}$ BASED ON TOTAL CELL VOLUME OF CLEAR LAKE WATER

$$\hat{X} \text{ (suspended solids, mg/l)} = 52.9 + 7.1 (\% \text{ secondary effluent added}), \quad (59)$$

$$\hat{X} \text{ (cells } \times 10^6/ml) = 2.22 + 0.252 (\% \text{ secondary effluent added}) \quad (60)$$

and

$$\hat{X} \text{ (total cell volume, } 10^6 \mu^3/ml) = 56.2 + 36.4 (\% \text{ secondary effluent added}), \quad (61)$$

The above equations give an indication of the amount of secondary effluent that can be added to Clear Lake water before specific limits for $\hat{X}$ are exceeded. For instance, if the limit for $\hat{X}$ is set at 100 mg suspended solids/l, the maximum addition of secondary effluent would be 6.6%. However, this does not indicate what the resultant algal cell concentration would be in Clear Lake if 6.6% of secondary effluent were added. It must be realized that the selection of limits for $\hat{X}$ would have to be derived empirically first because virtually no information exists on the relationship between the levels of biomass produced in algal assays, $\hat{X}$, and those produced in the identical water under natural conditions. In nature the conditions of temperature, light intensity, aeration, etc. vary greatly and are obviously not the same as those of algal assays. Furthermore, predation and settling phenomena tend to reduce natural standing crops, while this is prevented in assays. Therefore, the biomass levels in identical waters under natural and assay conditions should invariably result in lower concentrations of cells in the natural waters. The determination of the relationships between $\hat{X}$ and concentration of cells that would result in a specific receiving water for given concentrations of nutrients or wastes requires much additional research and field investigation. At present this appears to be a gigantic assignment.

It must be stressed that the control of eutrophication problems in a specific water system will require identification of the growth-limiting nutrients of the receiving water and the receiving water-waste mixture. Only then is it possible to know which nutrient, if any, and how much must be removed from the waste stream before discharge of wastes can occur without deleterious effects. Knowledge of the kinetic constants of various algae for the nutrient to be removed will be needed to decide on the amount of the nutrient that must be removed. Therefore, the development of the relationships between $\hat{X}$ and the concentration of wastes for a specific receiving water is merely a tool and not a final answer in eutrophication analysis.

Maximum Specific Growth Rate, Batch ($\hat{\mu}_b$). Addition of secondary effluent to Clear Lake water increased markedly the $\hat{\mu}_b$ above that of undiluted Clear Lake water as shown in Figure 28 and Table XII. With a concentration of 10% secondary effluent the increase in $\hat{\mu}_b$ was approximately 2.5 to 3.0 times (from 1.2 to 3.1 day⁻¹ for estimates based on cell counts and from 0.90 to 2.7 day⁻¹ for estimates based on total cell volume). Increased concentrations of secondary effluent did not increase further $\hat{\mu}_b$; in fact, $\hat{\mu}_b$ appeared to decrease. This decrease indicates that a concentration of secondary effluent above 10% is toxic to Scenedesmus capricornutum. This toxicity was not reflected in the $\hat{X}$ of the assays apparently because during the long incubation period the algae had sufficient time to reach their $\hat{X}$, although at a lower

rate than with the 10% secondary effluent cultures. The use of $\bar{X}$ alone as the algal growth parameter in assays would not detect such toxicity; however, the toxicity is quite apparent when growth-rate determinations ($\hat{\mu}_b$) are used.

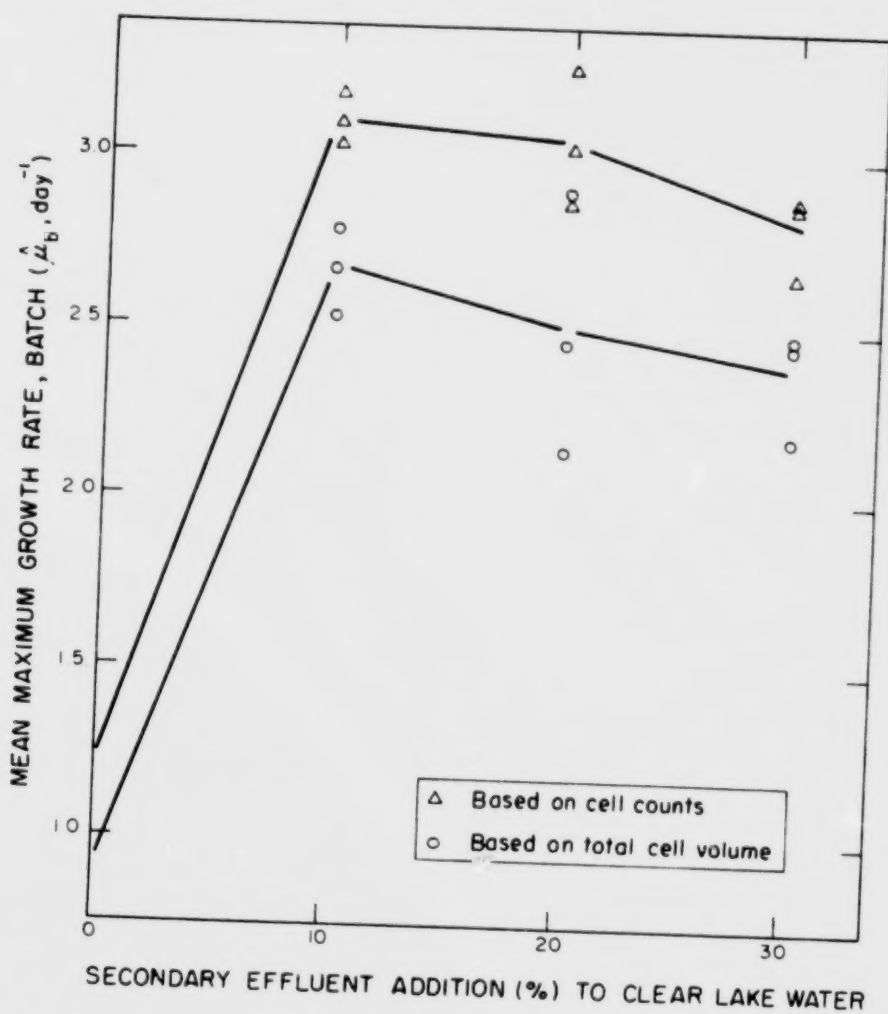


FIGURE 28. EFFECT OF SECONDARY EFFLUENT ON THE ALGAL GROWTH RATE OF CLEAR LAKE WATER

In the above case there would appear to be a greater chance of an algal bloom occurring in the presence of 10% secondary effluent than in the presence of 30% secondary effluent, assuming the toxicity is conservative. This is because the algal standing crop in a water is the sum total of the different rate reactions as shown in Figure 1, some of which increase and some which decrease the standing crop; therefore, a reduction in the growth rate, even through toxicity, likely would reduce the chances of an algal bloom occurring. However, induced toxicity is not a remedy for the eutrophication problem because as soon as the toxicant is suitably diluted or removed the growth rate would increase and a bloom would likely occur.

The detection of toxicity by the $\hat{\mu}_b$ measurement indicates the value of this algal response parameter. The measurement of $\hat{\mu}_b$ in batch assays must always be included.

Higher $\hat{\mu}_b$ values again were found for estimates based on cell counts as compared to estimates based on total cell volume. The reason for this difference is unknown. However, the behavior pattern of both estimates were similar.

Enrichment (Spiking) Studies on Clear Lake.

The spiking procedure outlined in Table X was followed. The cultures were inoculated and incubated under standard PAAP conditions for 16 days and the $\hat{X}$ estimates were made. Daily cell counts between the 2nd and 8th day were used for the calculation of $\hat{\mu}_b$. The results of the enrichments are summarized in Table C-V, Appendix C.

Maximum Cell Concentration ($\hat{X}$). The $\hat{X}$ estimates for the three biomass parameters are presented in Figure 29. None of the single nutrient spikes increased the $\hat{X}$ by more than a few percent. The N-P spike showed higher cell counts and total cell volume estimates than the control (no enrichment made) but the suspended solids value was lower. A small increase in all three biomass parameters was observed with the N-Fe spike whereas the P-Fe spike did not increase the $\hat{X}$ estimates above those of the control. Combined spiking of the N, P, and Fe increased the $\hat{X}$ slightly, whereas the addition of micronutrients to these three nutrients increased the $\hat{X}$ approximately twofold or more.

On basis of the above results it is difficult to determine which, if any, of N, P, or Fe was a growth-limiting nutrient. The significant stimulation of $\hat{X}$ by the addition of micronutrients indicated that a micronutrient was a secondary growth-limiting nutrient if not the primary growth-limiting nutrient.

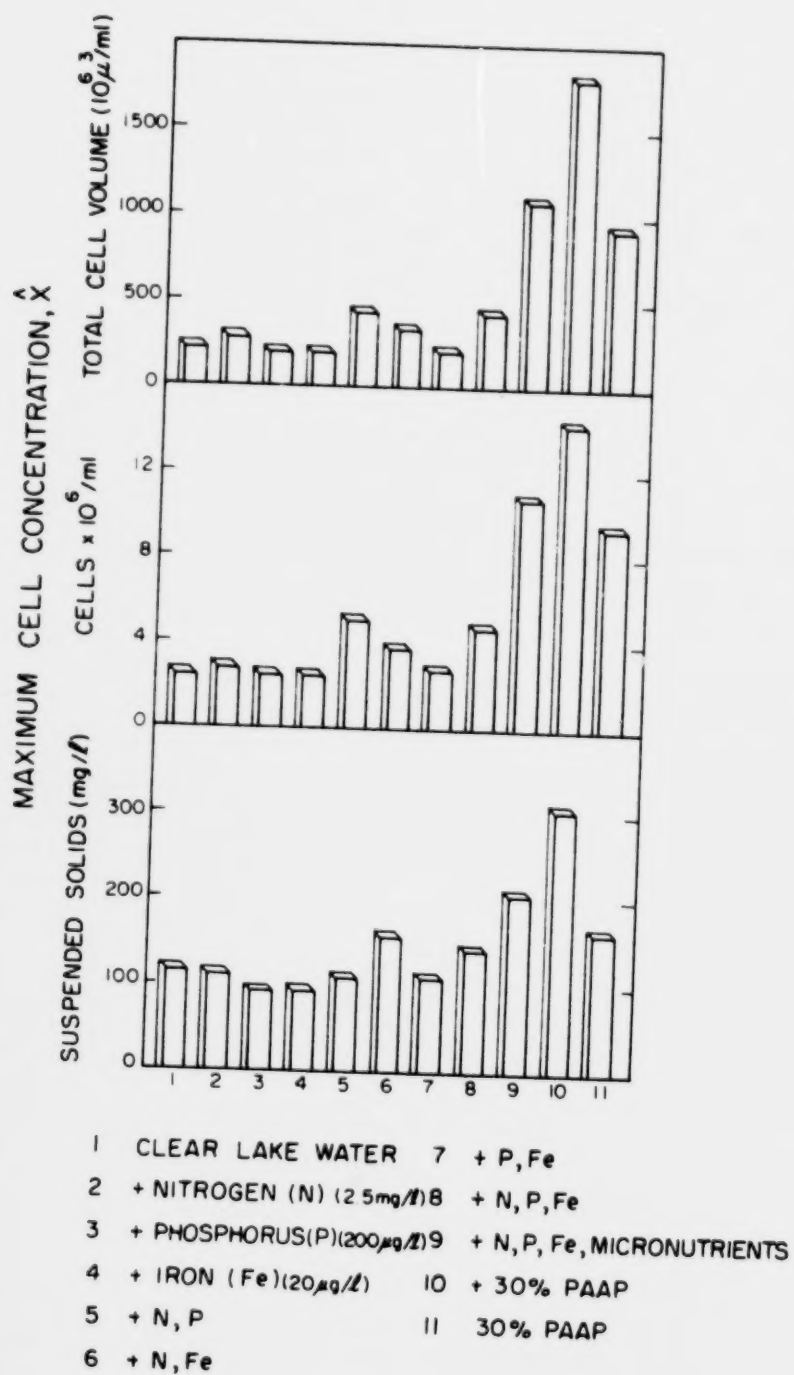


FIGURE 29. EFFECT OF ENRICHMENT (SPIKING) ON $\bar{x}$ OF CLEAR LAKE WATER

Maximum Specific Growth Rate, Batch ($\hat{\mu}_b$). The $\hat{\mu}_b$ values obtained in the spiking tests are summarized in Table XIV. The three single nutrient spikes increased the $\hat{\mu}_b$ whereas the N-P spike did not increase the $\hat{\mu}_b$. However, no clear pattern of behavior was observed because combined spikes such as N-P or N-P-Fe showed lower $\hat{\mu}_b$ values than for single nutrient spikes themselves. Addition of micronutrients showed a higher $\hat{\mu}_b$ than for single or combined spikes of N, P, or Fe. The pattern observed from the $\hat{\mu}_b$ determinations were similar to those for $\hat{X}$ estimates, and it is not possible to determine which nutrient was the primary limiting nutrient. However, the stimulation by micronutrients indicated that one of the micronutrients was likely limiting growth. If a micronutrient limited growth, the degree to which the chemicals used for spiking were contaminated by this micronutrient is important. Because all chemicals are usually contaminated by traces of other chemicals it is possible that the stimulation of $\hat{X}$ or $\hat{\mu}_b$ by other nutrient spikes could have been due to the presence of traces of a micronutrient in the nutrient spikes.

The results of the enrichment studies of Clear Lake water did indicate clearly which nutrient was primarily limiting growth. To determine this further studies would have to be undertaken. However, it appeared that one, or more, of the micronutrients was limiting growth. The possible role of micronutrients should not be overlooked as one of the important factors in controlling eutrophication of natural waters.

Sacramento River Studies. During the Sacramento River assays, control flasks of 30% PAAP medium produced an $\hat{X}$ (suspended solids) of 151.2 mg/l ($C_v = 7.4\%$) and an $\hat{X}$ (cells $\times 10^6/\text{ml}$) of 5.25 ($C_v = 27.0\%$). In addition, in Sacramento River water supplemented with 9% PAAP medium, an $\hat{X}$ (suspended solids) of 68.2 mg/l ($C_v = 5.4\%$) and an $\hat{X}$ (cells $10^6/\text{ml}$) of 4.43 ($C_v = 13.3\%$) were produced. Therefore, the inoculum appeared to be responsive to nutrients and growth occurred.

Maximum Cell Concentration ($\hat{X}$). Maximum cell concentration based on suspended solids, cell counts, and total cell volume were determined. The growth curves for all assays are presented in Table C-VI, Appendix C.

Undiluted Sacramento River water produced an $\hat{X}$ (suspended solids) of 15.2 mg/l ($C_v = 13.5\%$). Dilution of the water to 66.7% produced an $\hat{X}$ (suspended solids) of 10.4 mg/l ($C_v = 3.8\%$), and dilution to 33.3% produced an $\hat{X}$ (suspended solids) of 5.8 mg/l ($C_v = 2.9\%$). A highly significant ($p = 0.99$) correlation coefficient ($r = 0.97$, $n = 9$) was observed between the $\hat{X}$ (suspended solids) and the dilution of Sacramento River water as shown in Figure 30.

TABLE XIV

EFFECT OF NUTRIENT ENRICHMENT (SPIKES) ON MEAN
SPECIFIC GROWTH RATE, BATCH ($\hat{\mu}_b$)
OF CLEAR LAKE WATER

Addition To Clear Lake Water	Maximum Specific Growth Rate, Batch ($\hat{\mu}_b$)	
	Mean	Range
None	0.44	0.34 - 0.56
N ^a	0.69	0.53 - 0.85
P ^b	0.64	0.62 - 0.67
Fe ^c	0.70	0.64 - 0.77
N-P	0.46	0.44 - 0.48
N-Fe	0.79	0.56 - 1.03
P-Fe	0.74	0.64 - 0.84
N-P-Fe	0.54	0.39 - 0.69
N-P-Fe micronutrients ^d	0.83	0.82 - 0.84
30% PAAP	1.07	1.00 - 1.15
10% secondary effluent	0.51	0.46 - 0.60
Control (30% PAAP)	1.21	

^aFor all N spikes 2.5 mg N/l were added to samples.

^bFor all P spikes 200 μ g P/l were added to samples.

^cFor all Fe spikes 20 μ g Fe/l were added to samples.

^dMicronutrient spike identical in composition and concentration to that of 30% PAAP medium.

Undiluted Sacramento River water produced an $\hat{X}$ (cells $\times 10^6$ /ml) of 0.334 ($C_v = 14.5\%$). For 66.7% and 33.3% dilutions, $\hat{X}$ was 0.259×10^6 cells/ml ($C_v = 16.8\%$) and 0.134×10^6 cells/ml, respectively. There was a highly significant ($p = 0.99$) correlation coefficient ($r = 0.92$, $n = 9$) between the $\hat{X}$ (cells $\times 10^6$ /ml) and dilution of Sacramento River water (Figure 31). Similarly, $\hat{X}$ (total cell volume) values of 27.1, 20.0, and $9.1 \mu^3 \times 10^6$ /ml for respectively 100%, 66.7%, and 33.3% concentrations of Sacramento River water were highly significantly ($p = 0.99$) correlated ($r = 0.92$, $n = 9$) with the dilution of the water as shown in Figure 32.

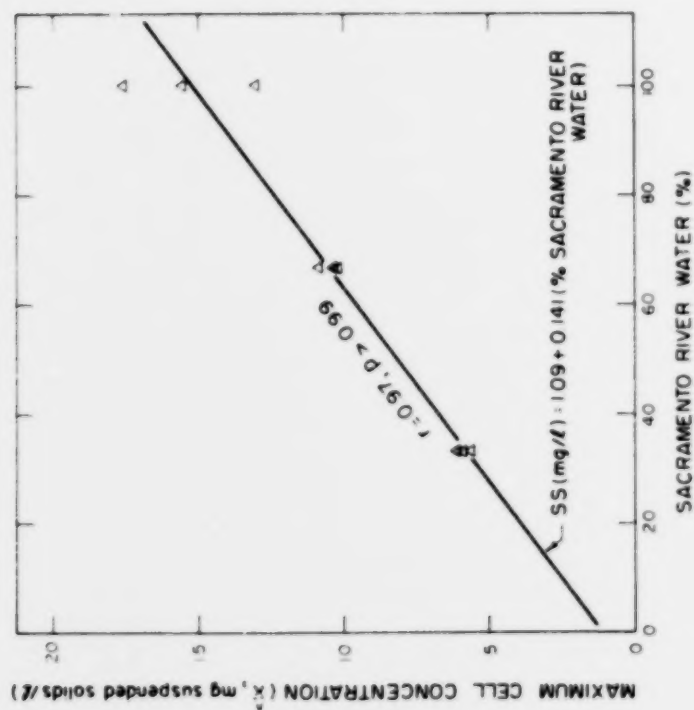


FIGURE 30 RELATIONSHIP BETWEEN DILUTION OF SACRAMENTO RIVER WATER AND $\bar{X}$ BASED ON SUSPENDED SOLIDS

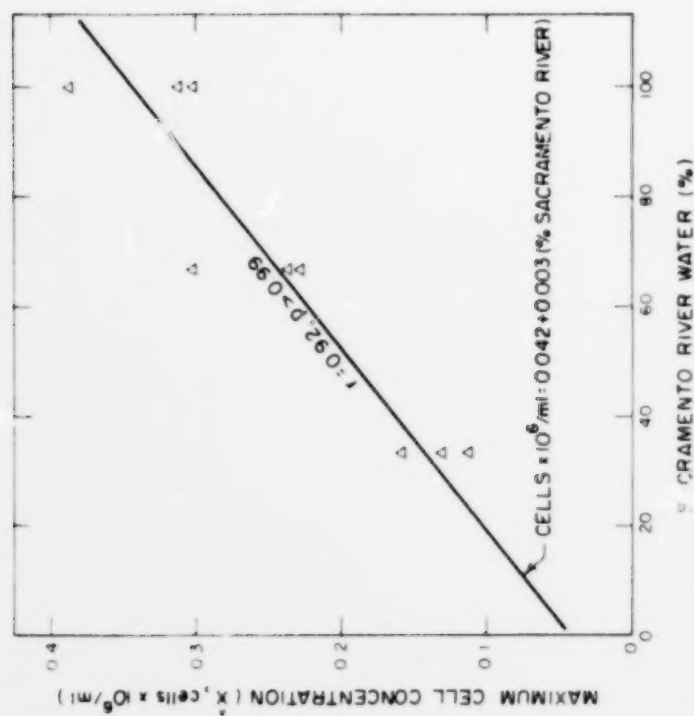


FIGURE 31 RELATIONSHIP BETWEEN DILUTION OF SACRAMENTO RIVER WATER AND $\bar{X}$ BASED ON CELL NUMBERS

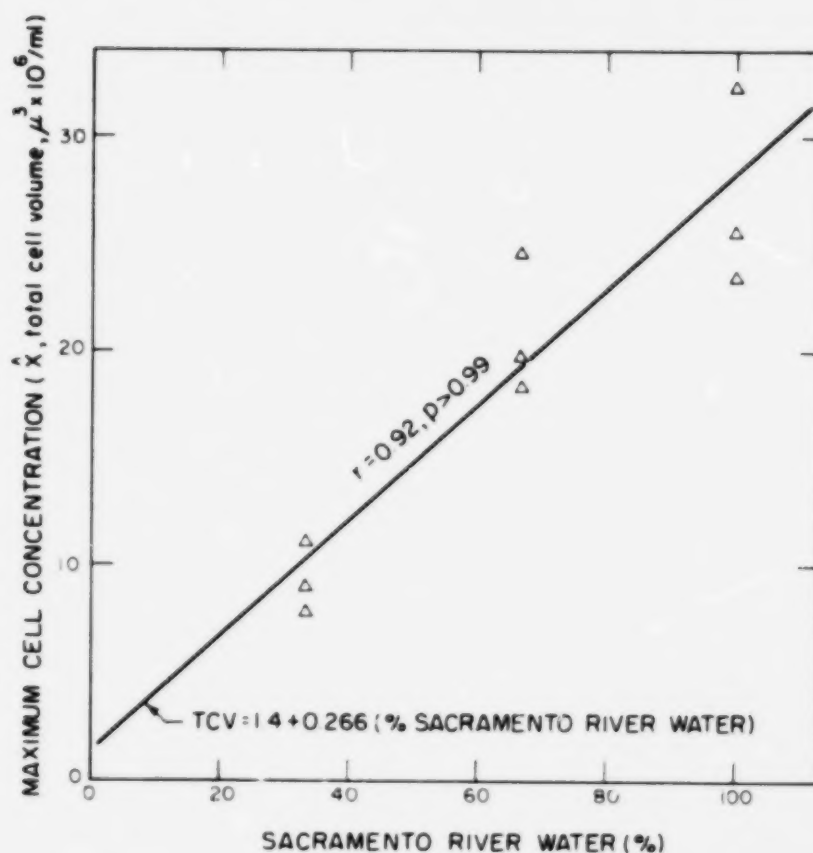


FIGURE 32. RELATIONSHIP BETWEEN DILUTION OF SACRAMENTO RIVER WATER AND $\hat{X}$ BASED ON TOTAL CELL VOLUME

As for Clear Lake samples, dilution of Sacramento River samples yielded $\hat{X}$ values proportional to the dilution factor. No evidence was obtained indicating the presence of toxicants. It appears that linear $\hat{X}$ responses can be obtained for dilution of natural samples having maximum values of $\hat{X}$ as low as 15 mg/l suspended solids or 0.334×10^6 cells/ml or $27.1 \mu^3 \times 10^6/\text{ml}$ total cell volume. Based upon these data it appears that the batch assay can then be used for assaying waters with nutrient concentrations as low as ($\text{NH}_3\text{-N} + \text{NO}_3\text{-N} = 133 \mu\text{g/l}$ and $\text{PO}_4\text{-P} = 34 \mu\text{g/l}$). The linearity in $\hat{X}$ responses indicates that the batch assay responds proportionally to the concentration of the $\hat{X}$ limiting nutrient and that the method can be used for screening natural samples. However, it should be noted that for zero concentrations of Sacramento River water, the $\hat{X}$ response ranged from 6% to 15% of the response for 100% Sacramento River water. No explanation for this anomaly is available.

The coefficients of variation for the different $\hat{X}$ estimates were all less than 18.5% which is acceptable. The same analyses for the Clear Lake estimates were 11% or below. The higher value for the Sacramento River assays is probably due to the fact that this water contained less nutrients and therefore resulted in lower $\hat{X}$ estimates. The precision of these estimates for Sacramento River water are judged to be adequate.

Maximum Specific Growth Rate, Batch ($\hat{\mu}_b$). The effect of dilution on the $\hat{\mu}_b$ of Sacramento River assays is shown in Figure 33. Dilution to 66.7% and 33.3% reduced the $\hat{\mu}_b$ (cell counts) by 37% and 79%, respectively, and the $\hat{\mu}_b$ (total cell volume) by 17% and 66%, respectively. Dilution of Sacramento River water had an appreciable effect on the $\hat{\mu}_b$ of the samples, probably because Sacramento River has a moderately low nutrient content, at least as compared to Clear Lake water. Hence dilution of Sacramento River water has a greater effect on the $\hat{\mu}_b$ than dilution of Clear Lake water because it brings the nutrient content of the diluted samples down into the range where growth is first order with respect to nutrient concentration.

The precision of the Sacramento River $\hat{\mu}_b$ estimates is presented in Table XV. Inspection of Table XV indicates that the coefficients of variation (C_v) for the estimates of $\hat{\mu}_b$ were markedly higher than for estimates of $\hat{X}$. In fact, the middle dilution of Sacramento River water (67%) had C_v 's of 43% and 57% for the $\hat{\mu}_b$ based upon cell count and cell volume, respectively. Most of the other values of the C_v were less than 20%. No adequate explanation is available for this lower level of precision except for the inherent variability in the growth rate of the batch assay and the fact that the counts used for computation of $\hat{\mu}_b$ were much less than for $\hat{X}$.

Utilization of Inorganic Nutrients During Assays. The concentration of inorganic nitrogen and phosphorus compounds in the Sacramento River samples used for these assays is summarized in Table XVI. All reported values are the means of either duplicate or triplicate analyses. Nitrogen was present in the undiluted Sacramento River water at a concentration of about 133 $\mu\text{g N/l}$; of this NO_3 and $\text{NO}_2\text{-N}$ contributed 81% and $\text{NH}_3\text{-N}$ 19%. Phosphate phosphorus was present at a concentration of 34 $\mu\text{g P/l}$. Sacramento River contained 60 $\mu\text{g N/l}$ and 362 $\mu\text{g P/l}$ less than the Clear Lake samples (Table XIII), which explains the lower $\hat{X}$ for the former. Sacramento River has a much lower nutrient content than Clear Lake.

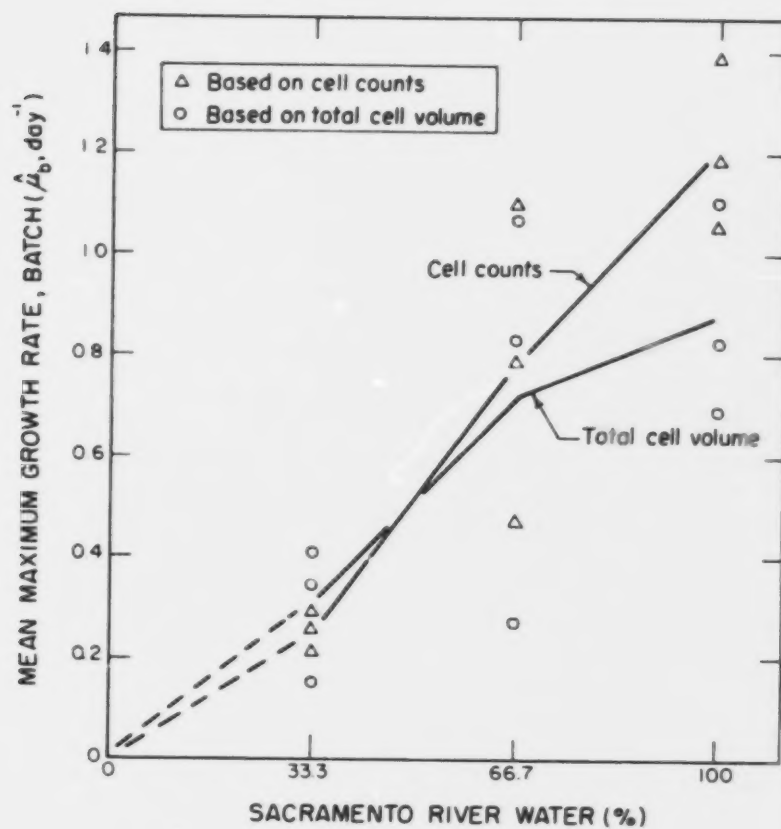


FIGURE 33. MAXIMUM BATCH GROWTH RATE ($\hat{\mu}_b$) IN SACRAMENTO RIVER WATER

TABLE XV
ALGAL GROWTH RATE ESTIMATES, $\hat{\mu}_b$,
FOR SACRAMENTO RIVER

Sample	Maximum Specific Growth Rate, $\hat{\mu}_b$ day ⁻¹ , Based On			
	Cell Count (10 ⁶ cells/ml)		Total Cell Volume (10 ⁶ μ^3 /ml)	
	Mean	C_v % ^a	Mean	C_v % ^a
100% SR ^b	1.21	13.8	0.87	23.9
67% SR	0.77	42.7	0.72	56.7
33% SR	0.25	15.8	0.30	42.5
SR + 30% PAAP ^c	3.17	20.3	2.33	14.0
30% PAAP	2.25	13.5	2.09	16.6
SR + 10% SE ^d	2.32	17.1	1.67	8.9
SR + 20% SE	2.50	3.7	1.92	4.5
SR + 30% SE	2.79	5.7	2.25	5.3

^a C_v % = Coefficient of variation = 100(standard deviation)/mean

^bSR = Sacramento River.

^cPAAP = Medium given in Tables I and II.

^dSE = Secondary effluents.

TABLE XVI
INORGANIC NITROGEN AND PHOSPHORUS CONCENTRATIONS IN SACRAMENTO RIVER WATER

Sacramento River Concentration	Initial Concentration, S_0 t_0 ($\mu\text{g}/\ell$)			Terminal Concentration, S_1 $t = 14$ days ($\mu\text{g}/\ell$)					
	$\text{NH}_3\text{-N}$	$\text{NO}_3 + \text{NO}_2\text{-N}$	$\text{PO}_4\text{-P}$	$\text{NH}_3\text{-N}$		$\text{NO}_3 + \text{NO}_2\text{-N}$		$\text{PO}_4\text{-P}$	
	Mean	Mean	Mean	Mean	C_v	Mean	C_v	Mean	C_v
100%	25.2	107.6	34.2	11.9	10.2	12.1	59.0	7.8	3.8
66.7%	16.6	75.0	22.1	12.6	b	5.7	14.3	5.2	18.5
33.3%	14.3	36.7	9.7	11.9	26.9	8.5	14.3	6.0	25.0

^a Coefficient of variation = 100 (standard deviation)/mean

^b Lack of replicates prevented estimate of coefficient of variation.

The use of filtration pretreatment in earlier studies of these two waters apparently resulted in a large removal of nutrients because the chemical analyses for both waters were similar [34]. The current findings indicate that filtration pretreatment must have removed substantial amounts of nutrients from the Clear Lake samples because these waters are considerably different in chemical composition. The initial inorganic N/P ratio of 3.9 suggests that nitrogen rather than phosphorus would be the limiting nutrient (if it is one of these) because phosphorus appears to be limiting at N/P ratios of more than 30 [34].

The accuracy of dilution of the Sacramento River water was illustrated by the highly significant ($p \geq 0.99$) correlation coefficients between either $\text{NH}_3\text{-N}$ or $\text{PO}_4\text{-P}$ or NO_3 and $\text{NO}_2\text{-N}$ concentrations and dilution concentration of the Sacramento River water. The correlation coefficients were 0.99, 0.99, and 0.86, respectively, for the relationships:

$$\text{NO}_3 \text{ and } \text{NO}_2\text{-N } (\mu\text{g}/\ell) = 3.3 + 1.05 (\% \text{ of Sacramento River water}) \quad (62)$$

$$\text{PO}_4\text{-P } (\mu\text{g}/\ell) = 0.37 (\% \text{ Sacramento River water}) - 2.5 \quad (63)$$

$$\text{NH}_3\text{-N } (\mu\text{g}/\ell) = 8.8 + 0.15 (\% \text{ Sacramento River water}) \quad (64)$$

During the assays at least 79% of the NO_3 and $\text{NO}_2\text{-N}$ was removed from solution in all dilutions of Sacramento River water. Terminal concentrations were $12.1 \mu\text{g N}/\ell$ or less. In the 33.3% samples apparently only 17% of the $\text{NH}_3\text{-N}$ was removed from solution; however, the amount of hydrolysis of nitrogenous particulate matter in the samples is not known, nor its effect on $\text{NH}_3\text{-N}$ concentrations. Because the samples contained a considerable amount of particulate matter, release of NH_3 through hydrolysis was a distinct possibility. Removal of $\text{PO}_4\text{-P}$ was at least 38% (33.3% dilution) and as high as 77% (66.7% and 100% cultures). However, the terminal $\text{PO}_4\text{-P}$ concentrations were about the same for all dilutions at a level of $5\text{-}8 \mu\text{g P}/\ell$. The amount of inorganic nutrient removal was not as high as that in the Clear Lake samples; the reason for this is not apparent.

Yield Coefficients for Nitrogen and Phosphorus. The Y_N values for the 100%, 66.7%, and 33.3% concentrations of Sacramento River water were respectively 140, 142, and 191. As with the Clear Lake assays, these values were much higher than expected. Porcella *et al.* [34] obtained Y_N values of 20 to 30 at low initial N/P ratios when nitrogen was the likely limiting nutrient. The values found in this study are at least four times greater than

those reported in the previous study. Inversion of the Y_N values suggests that the cells contained only 0.71%, 0.70%, and 0.52% nitrogen on a dry weight basis. Because these values seem low, it appears that hydrolysis of nitrogenous compounds in the particulate matter may have supplied nitrogen for cell synthesis. Based upon the data obtained on yield coefficients, it is not possible to conclude whether or not nitrogen is the growth-limiting nutrient.

The Y_P values of 577, 615, and 1582 for respectively the 100%, 66.7%, and 33.3% concentrations suggest that phosphorus might have been the growth-limiting nutrient because these values approached the Y_P values of approximately 900 to 1000 (when phosphorus was growth limiting) [34]. However, the fairly high terminal PO_4 -P values (5 to 8 $\mu\text{g P/l}$) and the fairly low initial PO_4 -P values suggest that if phosphorus was limiting growth, lower terminal values would have been expected.

As with the assay of Clear Lake water, determination of yield coefficients did not aid significantly in identification of the growth-limiting nutrients. The reason appears to be the tremendous variation in yield coefficients with batch assays. This finding also raises the significant question as to how satisfactory the batch assay can become in identifying the growth-limiting nutrient until a rational explanation is available for the variation in the yield coefficient.

Secondary Effluent Addition to Sacramento River Water.

Maximum Cell Concentration ($\hat{X}$). The $\hat{X}$ based on three different biomass parameters were increased by the addition of secondary effluent to Sacramento River water. The growth curves are summarized in Table C-VII, Appendix C.

In all instances a significant ($p \geq 0.99$) linear relationship was observed between the amount of secondary effluent added and the resulting $\hat{X}$. However, regression for $\hat{X}$ based upon cell counts and cell volume (Figures 34 and 35) indicate a lot of scatter in the data. No explanation is available for the variability in the data. These relationships are summarized in Figures 34, 35, and 36. The regression equations obtained were as follows:

$$\hat{X} \text{ (suspended solids, mg/l)} = 21.7 + 6.64 (\% \text{ secondary effluent}) \quad (65)$$

$$\hat{X} \text{ (cells } 10^6/\text{ml)} = 2.53 + 0.342 (\% \text{ secondary effluent added}) \quad (66)$$

$$\hat{X} \text{ (total cell volume, } 10^6 \mu^3/\text{ml)} = 202.2 + 27.14 (\% \text{ secondary effluent added}) \quad (67)$$

These relationships are similar to those obtained for Clear Lake water, i. e., the slopes for Clear Lake were respectively 7.1, 0.252, and 36.4 compared to the slopes of 6.64, 0.342, and 27.14 for the Sacramento River samples.

Using the foregoing relationships it is possible to estimate the amount of secondary effluent that can be added to the Sacramento River water before a specific limit is reached. For instance, 11.76% secondary effluent will produce an $\bar{X}$ of 100 mg suspended solids/l, compared to 6.6% in the case of Clear Lake. The difference for the two waters can be attributed to the lower nutrient content of the Sacramento River as indicated by the higher zero intercept of the Clear Lake equation (52.9 versus 21.7), and the lower slope for the Sacramento River (6.64 versus 7.1). However, as indicated previously how these numbers relate to the standing crop of plankton, $\bar{X}$ in either body of water is unknown. Moreover it is just this relationship that one needs to know to provide adequate control measures.

Maximum Specific Growth Rate, Batch ($\hat{\mu}_b$). The effects of addition of secondary effluent to Sacramento River water on $\hat{\mu}_b$ are shown in Figure 37. Addition of secondary effluent increased the $\hat{\mu}_b$ (based on cell counts or total cell volume). Unlike the Clear Lake sample, the addition of secondary effluent indicated no toxic effects on the algal growth rate of the Sacramento River water. Addition of secondary effluent to Sacramento River water will increase the possibility of an algal bloom developing and up to a concentration of 30% secondary effluent the growth rate appears to increase.

The data reported in Figure 37 indicate that there is something in secondary effluent (possibly trace elements) other than its nutrient content that is lacking in Sacramento River water. Once some of these limiting substances are added, the growth rate increases markedly (i. e., 0 to 10% secondary effluent). Thereafter from 10% to 30% the increase in growth rate is approximately linear with respect to the increase in concentration of nutrients added — at least up to a concentration of 30% secondary effluent. It would have been informative if a series of 1% or 2% secondary effluent assays had been included to assess this observation.

Growth rate measurements based on total cell volume measurements were again lower than those based on cell counts. The reason for this is not apparent. The precision of the $\hat{\mu}_b$ estimates for the addition of secondary effluent (Table XV) was satisfactory.

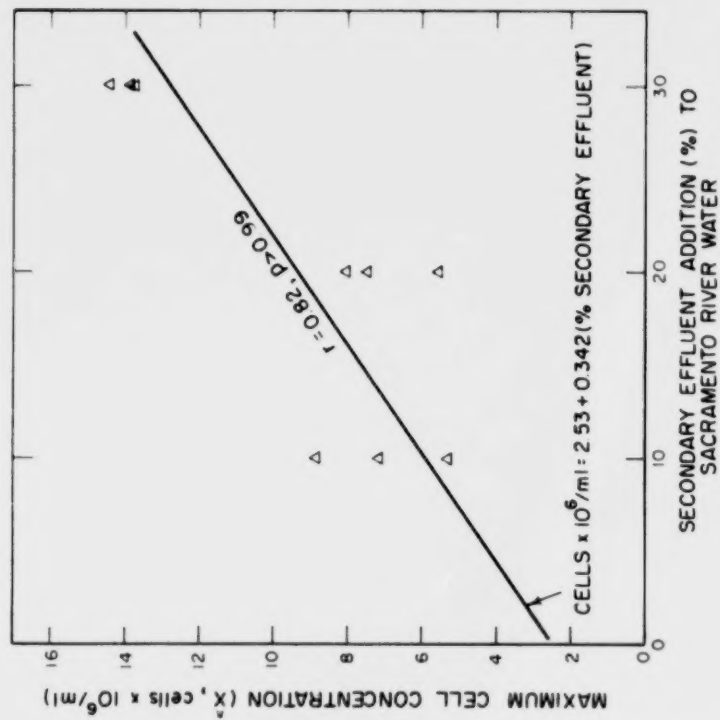


FIGURE 34. EFFECT OF SECONDARY EFFLUENT ADDITION ON $\bar{X}$ BASED ON CELL NUMBERS OF SACRAMENTO RIVER WATER

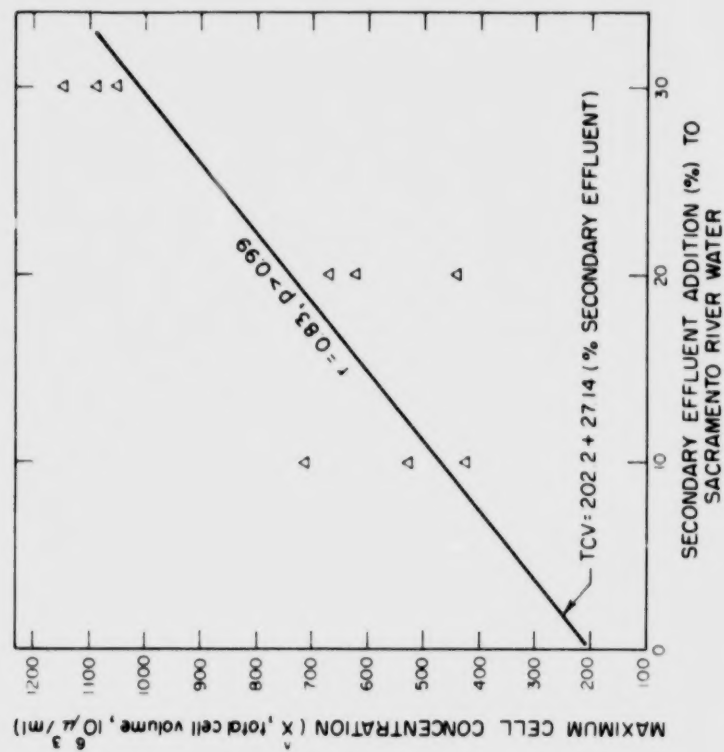


FIGURE 35. EFFECT OF SECONDARY EFFLUENT ADDITION ON $\bar{X}$ BASED ON TOTAL CELL VOLUME OF SACRAMENTO RIVER WATER

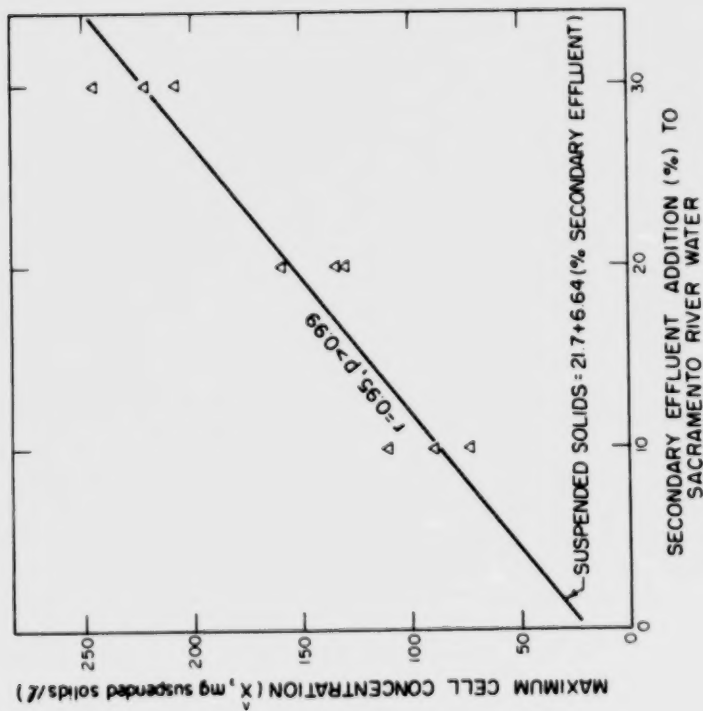


FIGURE 36. EFFECT OF SECONDARY EFFLUENT ADDITION ON $\bar{X}$ BASED ON SUSPENDED SOLIDS OF SACRAMENTO RIVER WATER

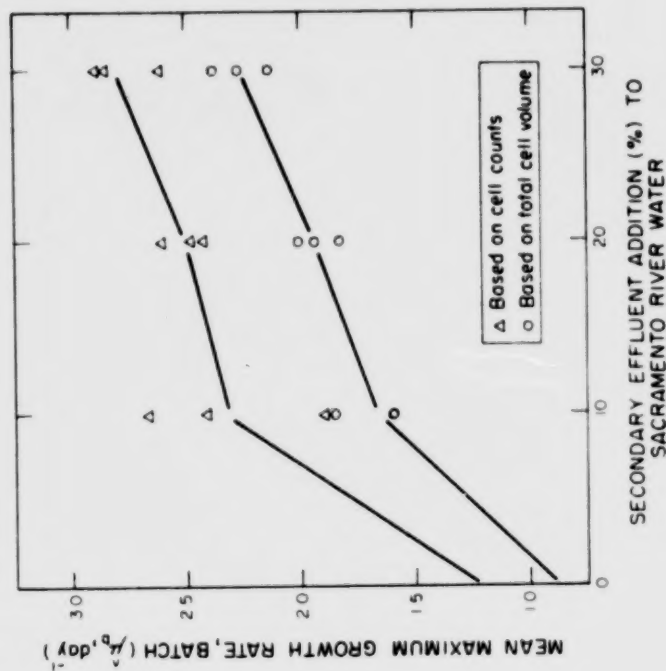


FIGURE 37. EFFECT OF SECONDARY EFFLUENT ADDITION ON $\bar{\mu}_b$ OF SACRAMENTO RIVER WATER

Enrichment (Spiking) Studies on Sacramento River Water.

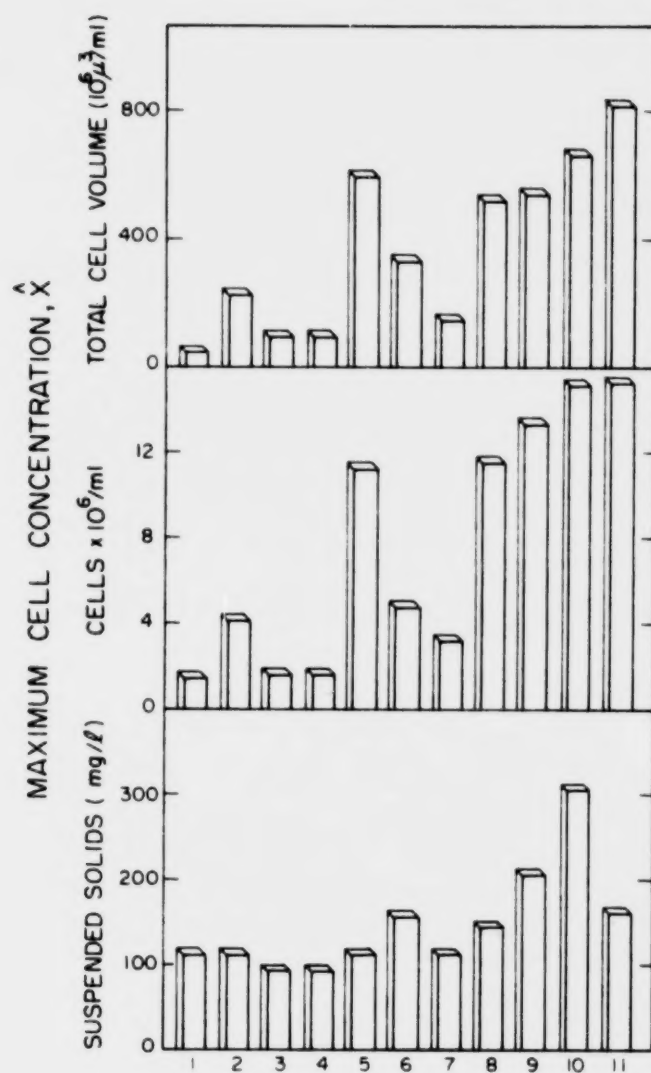
The spiking procedure outlined in Table X was followed. The cultures were inoculated and incubated under standard PAAP conditions for 16 days and the $\hat{X}$ estimates were made. Daily cell counts between the 2nd and 8th day were used for the calculation of $\hat{\mu}_b$. The results of the enrichments are summarized in Table C-VIII, Appendix C.

Maximum Cell Production ($\hat{X}$). The $\hat{X}$ estimates for the three biomass parameters are presented in Figure 38. All three biomass parameters gave essentially similar results. Of the single nutrient spikes only nitrogen appears to have increased the $\hat{X}$ above that of unspiked water. This suggests that nitrogen was the primary limiting nutrient in the water. The increased $\hat{X}$ of the nitrogen-phosphorus spike (whereas the nitrogen-iron and iron-phosphorus spikes had lower $\hat{X}$) suggests that phosphorus was the secondary limiting nutrient. This hypothesis is also supported by the high level of $\hat{X}$ of the nitrogen-phosphorus-iron spike. The absence of any significant increase in $\hat{X}$ between the nitrogen-iron-phosphorus spike and the nitrogen-phosphorus spike suggests that iron was not a limiting nutrient; however, one of the micronutrients appears to be limiting because addition of micronutrients produced the maximum value of $\hat{X}$. This supports the hypothesis cited earlier based upon examination of the $\hat{\mu}_b$ data shown in Figure 37.

Determination of the $\hat{X}$ values for different spikes of nutrients appears in this case to offer promise in the identification of limiting nutrients in a specific sample.

Maximum Specific Growth Rate, Batch ($\hat{\mu}_b$). The $\hat{\mu}_b$ values obtained in the spiking tests are summarized in Table XVII. The highest $\hat{\mu}_b$ values were obtained in the control (30% PAAP) and the N-P-Fe-micronutrient spikes, and were about 3.5 times as high as the unspiked Sacramento River water. The next highest growth rates were respectively for the Fe, P-Fe, N-P, N-Fe, and N-P-Fe spikes, whereas the N and P spikes had $\hat{\mu}_b$ values somewhat less than the Sacramento River water itself. The ranges of the $\hat{\mu}_b$ determinations (Table XV) showed that the differences were not due to variations in the $\hat{\mu}_b$ measurements but to real differences that existed between the spikes as illustrated in the summarized growth curves presented in Figure 39.

The only difference in composition between the N-P-Fe-micronutrient spike and the 30% PAAP medium spike was in the presence of macronutrients other than N and P. These macronutrients apparently were not growth rate limiting because they were present in sufficient quantities in the N-P-Fe-micronutrient spike. The



- | | |
|---|------------------------------|
| 1 SACRAMENTO RIVER WATER | 7 + P, Fe |
| 2 + NITROGEN (N) (2.5 mg/L) | 8 + N, P, Fe |
| 3 + PHOSPHORUS (P) (200 $\mu\text{g/L}$) | 9 + N, P, Fe, MICRONUTRIENTS |
| 4 + IRON (Fe) (20 $\mu\text{g/L}$) | 10 + 30% PAAP |
| 5 + N, P | 11 30% PAAP |
| 6 + N, Fe | |

FIGURE 38. EFFECT OF ENRICHMENT (SPIKING) ON THE $\bar{X}$ OF SACRAMENTO RIVER WATER

TABLE XVII
EFFECT OF NUTRIENT ENRICHMENT (SPIKES) ON
MEAN SPECIFIC GROWTH RATE, BATCH (μ_b)
OF SACRAMENTO RIVER WATER

Addition To Sacramento River Water	Maximum Specific Growth Rate, Batch (μ_b)	
	Mean	Range
None	0.61	0.44 - 0.83
N ^a	0.29	0.25 - 0.34
P ^b	0.41	0.37 - 0.45
Fe ^c	1.95	1.76 - 2.15
N-P	1.57	1.55 - 1.59
N-Fe	1.50	1.49 - 1.51
P-Fe	1.75	1.13 - 2.38
N-P-Fe	1.38	1.42 - 1.33
N-P-Fe-micronutrients ^d	2.02	1.75 - 2.30
30% PAAP	2.02	1.70 - 2.38
10% secondary effluent	0.62	0.43 - 0.76
Control (30% PAAP)	2.21	

^aFor all N spikes 2.5 mg N/l were added to sample.

^bFor all P spikes 200 μ g P/l were added to sample.

^cFor all Fe spikes 20 μ g Fe/l were added to sample.

^dMicronutrient spike identical in composition and concentration to that of 30% PAAP medium.

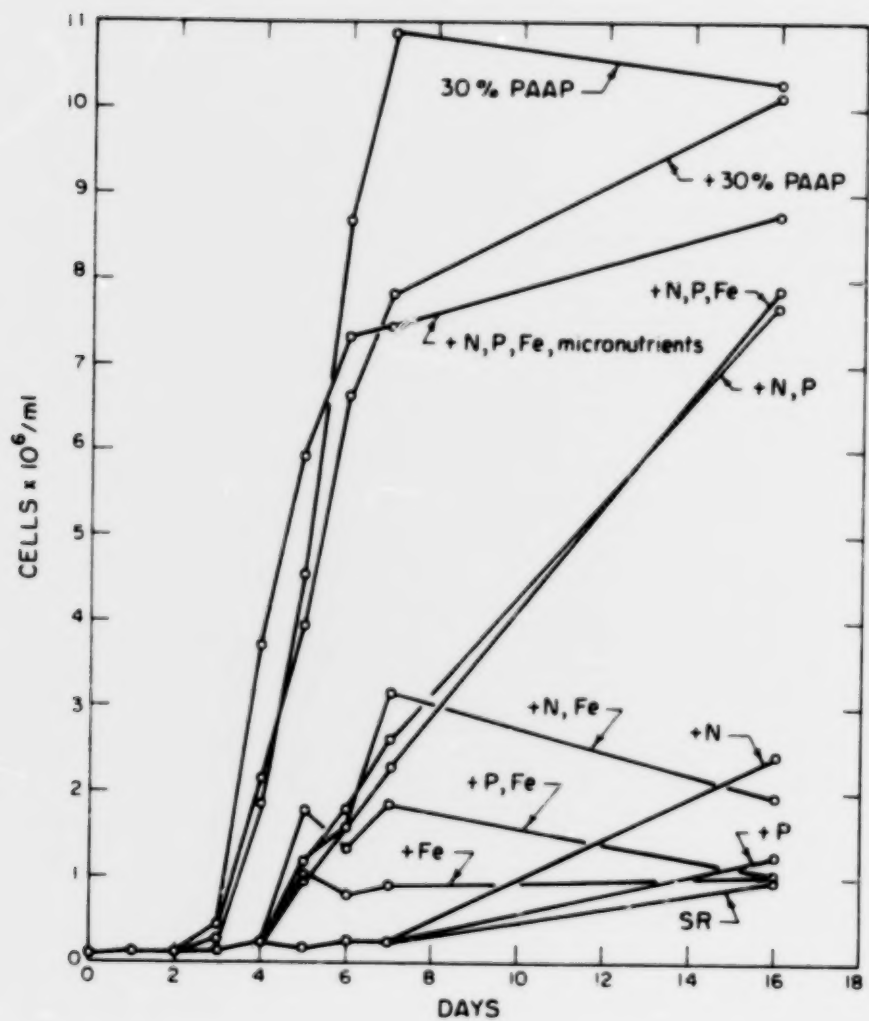


FIGURE 39. GROWTH CURVES OF ENRICHMENTS (SPIKES) OF SACRAMENTO RIVER WATER

differences in the $\hat{\mu}_b$ stimulation between the N-P-Fe-micronutrient spike and the rest of the spikes appear to be due to the presence of micronutrients. It is strongly indicated that the $\hat{\mu}_b$ of the Sacramento River water was limited by micronutrients. Because the experiment was primarily aimed at investigating and evaluating the spiking of N, P, and Fe, micronutrients were lumped together and not investigated singly. This is unfortunate, as it would have been most informative to have a separate spike of micronutrients. However, laboratory chemicals are usually contaminated with small amounts of other chemicals; therefore, the growth stimulation of spikes other than micronutrients could have been due to the trace contaminants (micronutrients) and not to the nutrients or other chemicals. The role of Fe might be considered in this connection; in all cases where Fe was one of the spikes, higher $\hat{\mu}_b$ values (compared to the unspiked water) were observed. It is not clear if Fe per se or a contaminant of micronutrient(s) in the FeCl_3 solution induced these increases.

The foregoing observations have considerable practical implications. First, if conclusions were drawn only from the $\hat{X}$ values, nitrogen would have been identified as the limiting nutrient of concern. However, the fact that nitrogen did not increase (a seeming decrease was noted) the $\hat{\mu}_b$ of the water indicated that even though a higher $\hat{X}$ would be produced in the presence of nitrogen, this would not occur at a higher rate. Therefore, if the algal standing crop is determined by different growth rates which either increase or decrease the standing crop (Figure 1), addition of nitrogen would not cause an increase in algal concentration unless the addition of nitrogen reduces the rates of the decreasing factors.

Second, the importance of nutrients other than N, P, and Fe is illustrated by these findings. The prime importance attached to nutrients such as N and P should not result in overlooking other important growth-limiting nutrients or factors such as micronutrients.

Third, the different patterns observed with $\hat{\mu}_b$ and $\hat{X}$ measurements upon spiking of nutrients illustrates the importance of $\hat{\mu}_b$ measurements. It is not necessary to contend that $\hat{\mu}_b$ measurements are more important than $\hat{X}$ measurements, but the ability of these measurements to indicate toxic and other factors which affect growth rates and which are not reflected in $\hat{X}$ values warrant their use and further investigation.

Present Status of Batch Culture Assays

The results of this investigation, the previous report [34], and reports from other evaluation laboratories [55-77] have shown that the batch

culture assay, as presently constituted and presented in this report, is an adequate algal assay in some respects but not in others. For instance, the algal responses $\hat{X}$ and $\hat{\mu}_D$ can be estimated fairly precisely ($C_v \leq 15\%$) in synthetic media (also between laboratories) and in natural water samples. Therefore, it is possible to determine the amount of algal cells that can be produced under optimum conditions in a specific water and also the rate at which they are produced. Furthermore, the algal response in terms of $\hat{X}$, providing that the necessary precautions are met, appears to be related linearly to the concentration of the $\hat{X}$ limiting nutrient present, thereby allowing the determination of the relationship between the addition of a wastewater (such as secondary effluent) and the maximal amount of algal cells, $\hat{X}$, that can be produced in the laboratory. The batch culture assay also has the advantages of being relatively simple to perform and is fairly economical. It seems to be adequate for assaying waters of moderately to highly enriched nutrient content and toxicity of wastes can be determined if $\hat{\mu}_D$ measurements are made.

The less satisfactory aspects of the batch assay are centered around both theoretical and practical concepts. Batch cultures by virtue of their inherent transient unbalanced growth characteristics with resultingly wide variations in nutrient and biomass levels, are not ideal for growth studies where these involve the definition of nutrient-growth rate relationships. For example, the use of batch culture data to determine algal growth kinetics using different growth models was unsatisfactory [34]. The identification of growth-limiting nutrients by batch assays is still in an unsatisfactory state. The use of yield coefficients appears to be limited by the gross variations in these coefficients while spiking (enrichment) techniques are not developed to the extent where a particular method can be recommended. However, through further research a satisfactory method will probably be developed.

One of the greatest limitations of the batch culture at present is the absence of a clear rational framework in which assay results may be applied in solving eutrophication problems. Merely obtaining the $\hat{X}$ and $\hat{\mu}_D$ responses of a test algal species in a natural water sample or knowing that the addition of a wastewater will increase this $\hat{X}$ or $\hat{\mu}_D$, still in no way indicates what will happen specifically in a natural system even for the same algal species. Many other factors influence the algal standing crop and all of these are reflected by various rates. To extrapolate batch assay results to real eutrophication problems will necessitate knowing the identity of the growth-limiting nutrient, the growth kinetics of various algae for this nutrient, the interaction between environmental factors and nutrients in regulating algal concentrations in nature, etc. For instance, once the identity of the growth-limiting nutrient has been established the decision on the amount of the nutrient, if any, that must be removed can only be decided on the basis of the growth kinetics of algae for this nutrient, i. e., how does the nutrient regulate the algal growth rate, how much algal mass is

produced per unit mass of the nutrient, etc. The development of the rational framework for the application of batch bioassay results must be developed through research on algal growth kinetics and the inter-relationships of various natural rate factors that determine the algal standing crop.

The present status of the batch assay is only that of a screening device for monitoring the general ability of waters to support the growth of algae and how this ability is grossly affected by wastes, nutrients, or other growth factors.

CONTINUOUS CULTURE STUDIES

Continuous Culture Growth Kinetics Under Phosphorus Growth Limitations

Twelve chemostats of the type shown in Figure 16 were used for continuous culture studies. They were operated under a phosphorus-limiting condition (i. e., constant cultural environment and all nutrients other than phosphate in excess) in a constant temperature room ($24^{\circ}\text{C} \pm 1^{\circ}\text{C}$). Five different residence times were used for each chemostat ($\theta = 0.7$ to ∞ days) during the study period (55 days). Table XVIII summarizes the conditions for the chemostat assays showing the type of feed media, the N/P ratio of the feed, the phosphorus content of the feed (S_0), the residence time, and the number of replicate chemostats.

Phosphorus as the Limiting Nutrient. In the PAAP medium (Tables I and II), nitrogen is considered to be in excess because the N/P ratio is 22.6. McGauhey *et al.* [30] reported that phosphorus usually was the rate-limiting nutrient when the N/P ratio was much greater than 15.

Porcella *et al.* [34] reported that phosphorus was probably the limiting nutrient in the PAAP medium from chemostat studies, and their batch results indicated that phosphorus was the only limiting nutrient at a N/P ratio ≥ 40 . In this chemostat study the phosphorus content of the medium was reduced to effect N/P ratios between 40 and 115. This was done to ensure that phosphorus was the sole limiting nutrient. The linear relationships between steady state biomass (suspended solids) and phosphorus uptake at different residence times as shown in Figure 40 indicates that phosphorus was the only limiting nutrient.

Steady State Consideration. The changes in biomass (absorbance) with time for all 12 chemostats are shown in Figures 41, 42, 43, and 44, along with input phosphorus concentrations and operating hydraulic residence times. (The operational data from the chemostat studies

TABLE XVIII
CONDITIONS FOR CHEMOSTAT ASSAYS

Medium	Average N/P Ratio	Average Residence Time, days	No. of Chemostats
50% PAAP medium with $S_{OP} = 66 \mu\text{g}/\text{l}$ as P	110 69.3 & 111 102 104.8 105	0.878 1.31 2.82 4.77 ∞	3
50% PAAP medium with $S_{OP} = 100 \mu\text{g}/\text{l}$ as P	72.5 59.5 & 71.9 68.2 67.7 67.7	0.928 1.34 2.88 4.97 ∞	3
50% PAAP medium with $S_{OP} = 135 \mu\text{g}/\text{l}$ as P	54.4 52.7 52.0 51.2 51.2	0.889 1.33 2.72 4.81 ∞	3
50% PAAP medium with $S_{OP} = 170 \mu\text{g}/\text{l}$ as P	43.1 41.8 41.4 40.8 40.8	0.877 1.31 2.70 5.12 ∞	3

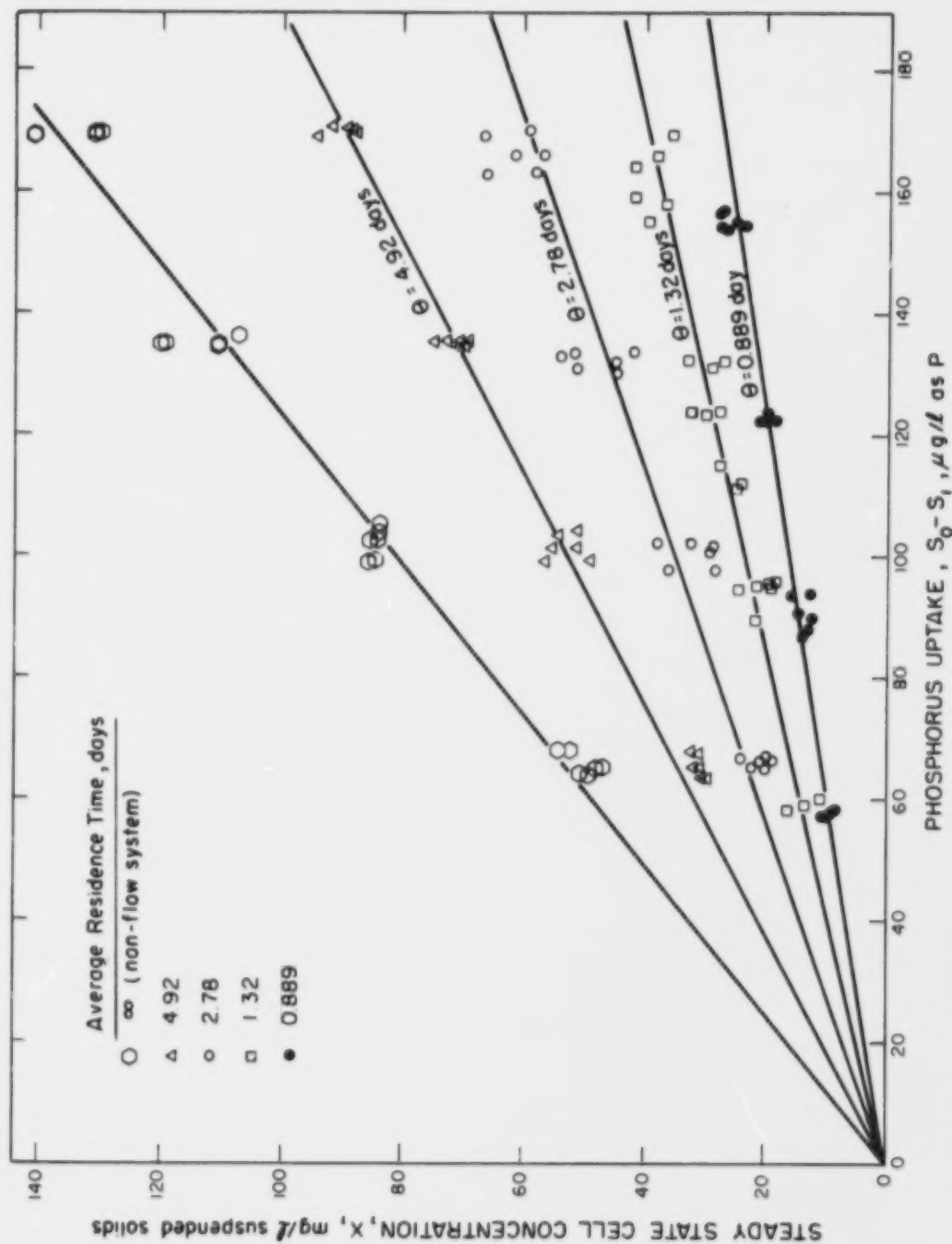


FIGURE 40. RELATIONSHIP BETWEEN CELL BIOMASS AND PHOSPHORUS UPTAKE FOR DIFFERENT RESIDENCE TIMES

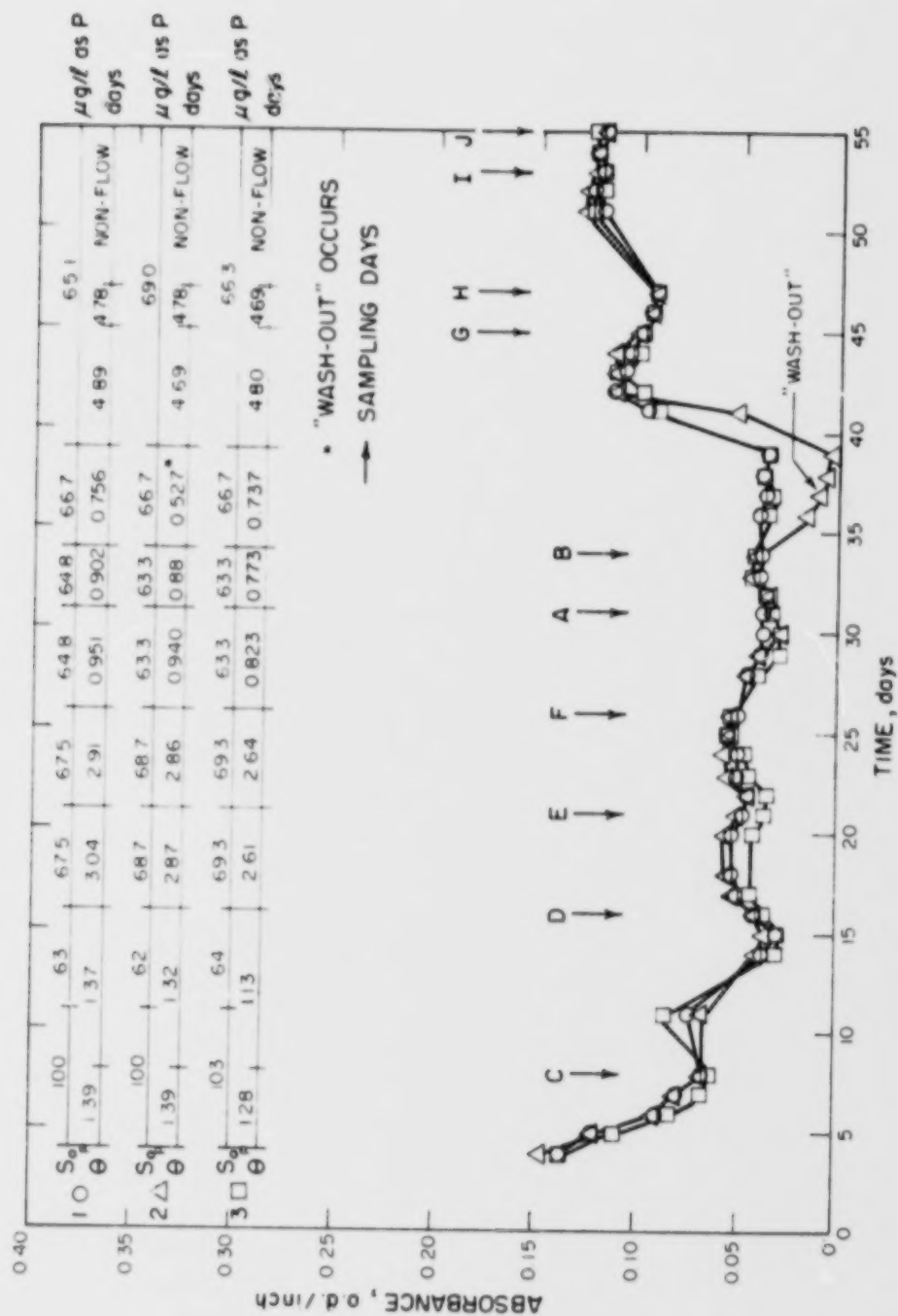


FIGURE 41. OPERATION OF CHEMOSTATS 1, 2, AND 3

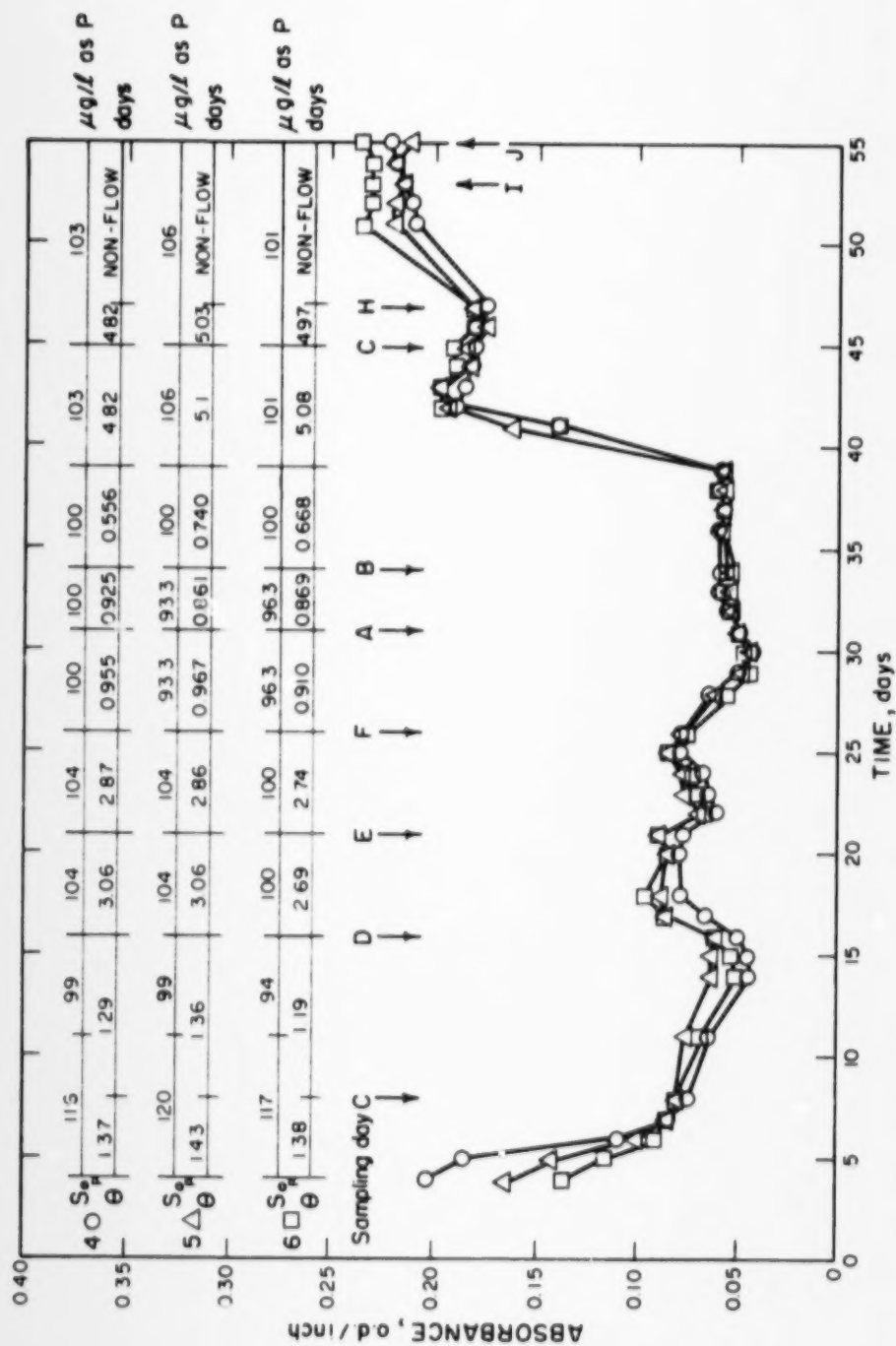


FIGURE 42. OPERATION OF CHEMOSTATS 4, 5, AND 6

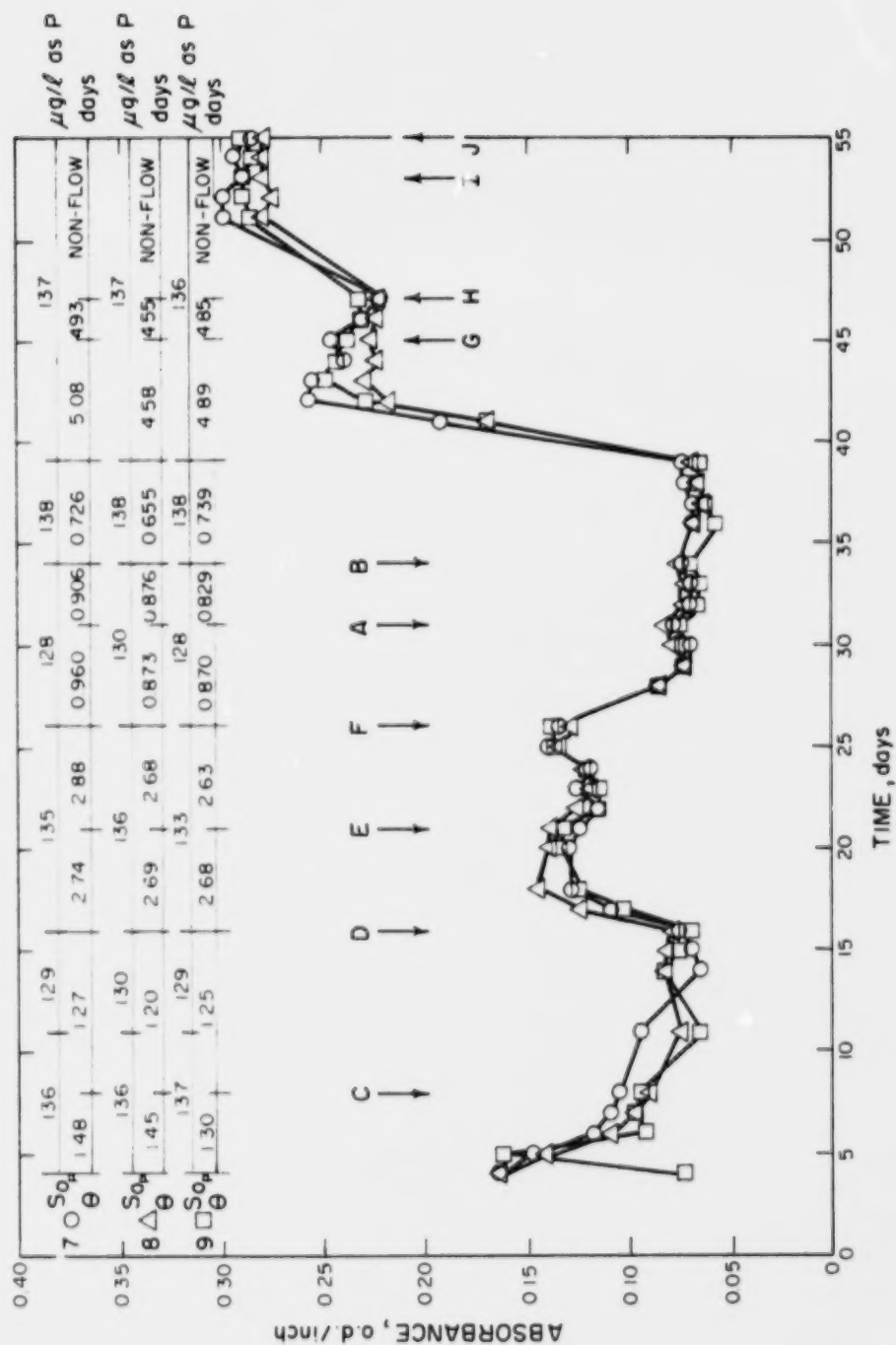


FIGURE 43. OPERATION OF CHEMOSTATS 7, 8, AND 9

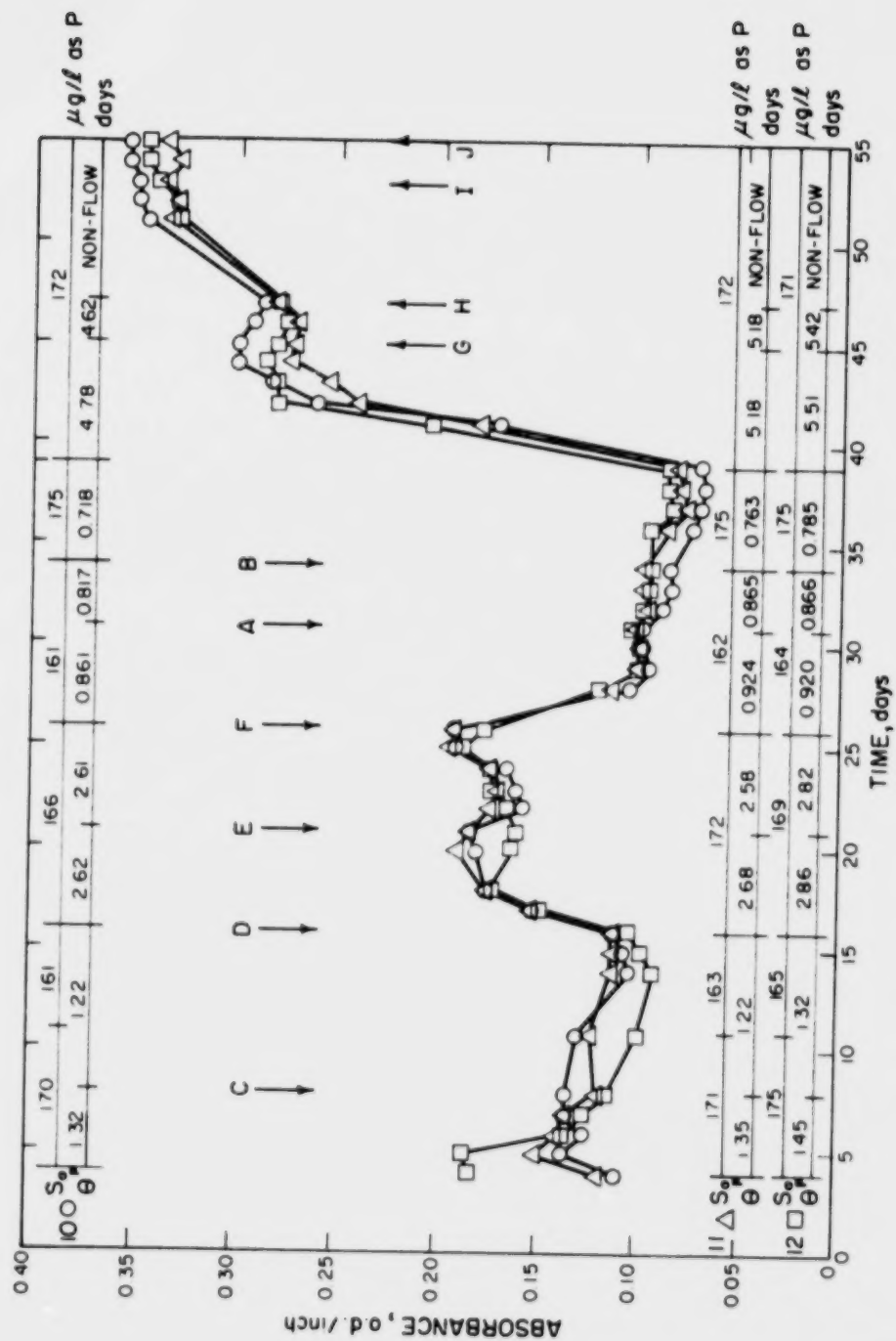


FIGURE 44. OPERATION OF CHEMOSTATS 10, 11, AND 12

are tabulated in Appendix D.) Reasonable steady states were approached at different phosphorus input levels and different hydraulic residence times. These operation curves show clearly that the steady state biomass level varies not only with the input phosphorus concentration but also with the hydraulic residence time (mean cell age, or growth rate). Figure 40 also indicates that the net yield coefficient (i. e., $Y_n = X_1/(S_0 - S_1)$ or slopes in Figure 40) is some function of residence time instead of a constant coefficient as discussed previously in the theory section.

Attempts were made to keep the chemostat cultures in a pure culture condition throughout the whole study period. Microscopic observations were carried out frequently to check the purity of the cultures. Two chemostats (Chemostat #9 and #12) were found to be contaminated with a very small population of bacteria during the later study period (Chemostat Run 1-9 and 1-12). However, no significant effect on steady state cell concentration and limiting-nutrient concentration in chemostat was observed.

Precision of Chemostat Assays. All chemostat assays were done with three replicate culture systems. The overall precision based on suspended solids and orthophosphate were estimated and reported in Table XIX. The suspended solids measurement is more precise than orthophosphate analysis (Table III), especially at very low orthophosphate concentration (0.45 to 6.0 $\mu\text{g P/l}$). Consequently, the variance due solely to the chemostat assay procedure based upon orthophosphate is higher than that based upon suspended solids. A coefficient of variation of 6.96% for the chemostat assay procedure was obtained with respect to suspended solids measurements, X_1 , while an average of 13.2% was obtained based on orthophosphate analysis (S_1). It appears that an average of coefficient of variation of about 10% was observed for the chemostat assay compared to about 15% of batch bioassays reported by Porcella *et al.* [34].

Summary of Chemostat Results. The results obtained with the chemostat experiments in which phosphorus alone constituted the growth-limiting nutrient are summarized in Tables XX through XXIV. All data used for kinetic analysis were taken at times when a steady state was reached (shown with arrows in Figures 41, 42, 43, and 44). Included in Tables XX through XXIV are the steady state cell concentration (X_1), the hydraulic residence time (θ), the concentration of phosphorus in the influent (S_0) and in the effluent (S_1), the calculated net yield coefficient ($Y_n = X_1/(S_0 - S_1)$), the fraction of phosphorus excessively taken up, (f_e), the nutrient removal velocity, ($q = (S_0 - S_1)/(X_1\theta)$), the amount of excess nutrient uptake, S_e , and the cell phosphorus constant, A .

TABLE XIX
PRECISION OF CHEMOSTAT BIOASSAY PROCEDURE

Parameter	Overall Precision of Chemostat Bioassays ^a			Precision of Analytical Methods ^b		Precision of Chemostat Procedure
	No. of Cases	$\bar{X}_o^f$ Mean	$C_{v_o}^c$	$\bar{X}_m^g$ Mean	$C_{v_m}^d$	$C_{v_a}^e$
Suspended Solids mg/l	120	37.0	6.99	16.5	1.30	6.96
PO ₄ -P μg/l P	48	4.65	13.3	5.04	7.62	10.40
	72	0.853	23.0	0.675	20.9	16.0

^aVariance of chemostat assay (overall) = variance of method plus variance of chemostat test technique.

^bData taken from Table III.

^c C_{v_o} = coefficients of variation (overall) determined from triplicate chemostats.

^d C_{v_m} = coefficients of variation determined from the precision of methods (Table III).

^e C_{v_a} = the average coefficient of variation contributed only by the chemostat procedure as expressed by:

$$C_{v_a} = \sqrt{C_{v_o}^2 - C_{v_m}^2 \frac{\bar{X}_m^2}{\bar{X}_o^2}}$$

^f $\bar{X}_o$ = arithmetic mean of the results bioassay (overall) response.

^g $\bar{X}_m$ = arithmetic means of results of analytical methods.

TABLE XX
SUMMARY OF CHEMOSTAT RUNS A AND B

PO ₄ -P										
Chemostat Run No.	θ_c Cell Age days	X_1 Suspended Solids mg/l	S_0	S_1	$S_0 - S_1$	$Y_n = \frac{X_1}{S_0 - S_1}$	$A = \frac{S_0 - S_1}{X_1}$	$q = \frac{S_0 - S_1}{X_1 \theta}$	S_e	f_e
			$\mu\text{g/l P}$	$\mu\text{g/l P}$	$\mu\text{g/l P}$	$\frac{\text{mg-cell}}{\text{mg P}}$	$\frac{\mu\text{g P}}{\text{mg-cell}}$	$\frac{\mu\text{g P}}{\text{mg-cell-day}}$	$\mu\text{g/l P}$	Fraction
A - 1	0.951	11.1	64.8	4.80	60	185	5.41	5.68	46.2	0.770
A - 2	0.940	11.7	63.3	5.10	58.2	201	4.97	5.29	43.7	0.751
A - 3	0.823	11.4	63.3	5.25	58.0	197	5.09	6.18	43.8	0.755
A - 4	0.955	15.7	100.0	5.40	94.6	166	6.03	6.31	75.1	0.796
A - 5	0.967	13.5	93.3	4.80	88.5	153	6.56	6.78	71.7	0.810
A - 6	0.910	14.7	96.3	5.1	91.2	161	6.20	6.82	72.9	0.799
A - 7	0.960	19.2	128.0	4.95	123	156	6.41	6.67	99.0	0.805
A - 8	0.873	20.1	130.0	4.65	125	161	6.22	7.12	100.0	0.800
A - 9	0.870	18.3	128.0	5.10	123	149	6.72	7.73	100.2	0.815
A - 10	0.867	23.8	161.0	5.85	155	154	6.51	7.51	125.4	0.809
A - 11	0.924	25.8	162.0	5.70	156	165	6.05	6.54	124.0	0.795
A - 12	0.920	27.5	164	6.0	158	174	5.75	6.25	123.9	0.784
B - 1	0.902	10.0	64.8	5.1	59.7	168	5.97	6.62	47.2	0.791
B - 2	0.880	12.9	63.3	5.1	58.2	222	4.51	5.13	42.2	0.725
B - 3	0.773	11.2	63.3	4.95	58.3	192	5.20	6.73	44.4	0.762
B - 4	0.905	13.5	100	5.1	94.9	142	7.03	7.77	78.1	0.823
B - 5	0.861	14.4	93.3	5.4	87.9	164	6.10	7.09	70.0	0.796
B - 6	0.869	13.9	96.3	5.25	91.0	153	6.54	7.53	73.7	0.810
B - 7	0.926	21.1	128	5.25	123	172	6.25	6.30	96.8	0.787
B - 8	0.876	20.0	130	5.4	125	160	5.83	7.13	100.1	0.801
B - 9	0.829	20.3	128	5.25	123	165	6.06	7.31	97.8	0.795
B - 10	0.817	26.4	161	5.85	155	170	5.87	7.19	122.1	0.788
B - 11	0.865	27.4	162	6.45	155	170	5.66	6.54	121.1	0.781
B - 12	0.866	27.6	164	6.3	158	175	5.72	6.61	123.7	0.783

TABLE XXI
SUMMARY OF CHEMOSTAT RUNS C AND D

Chemostat Run No.	θ_c Cell Age days	X_1 Suspended Solids mg/l	PO ₄ -P							
			S_0	S_1	$S_0 - S_1$	$Y_n = \frac{X_1}{S_0 - S_1}$	$A = \frac{S_0 - S_1}{X_1}$	$q = \frac{S_0 - S_1}{X_1 \theta}$	S_e	f_e
			$\mu\text{g/l P}$	$\mu\text{g/l P}$	$\mu\text{g/l P}$	$\frac{\text{mg-cell}}{\text{mg P}}$	$\frac{\mu\text{g P}}{\text{mg-cell}}$	$\frac{\mu\text{g P}}{\text{mg-cell-day}}$	$\mu\text{g/l P}$	Fraction
C - 1	1.39	18.8	100	3.3	96.7	194	5.14	3.70	73.3	0.758
C - 2	1.39	19.5	100	4.5	95.5	204	4.89	3.52	71.2	0.746
C - 3	1.28	18.6	103	3.3	96.7	192	5.20	4.06	73.6	0.761
C - 4	1.37	25.0	116	3.6	112	223	4.48	3.27	80.9	0.722
C - 5	1.43	27.2	120	3.9	116	234	4.26	2.98	82.2	0.709
C - 6	1.38	24.6	117	3.9	113	218	4.59	3.33	82.4	0.729
C - 7	1.48	33.1	136	3.0	133	249	4.02	2.71	91.9	0.691
C - 8	1.45	29.1	136	4.5	132	221	4.54	3.13	95.8	0.726
C - 9	1.30	27.0	137	3.9	133	203	4.93	3.79	99.4	0.747
C - 10	1.32	41.2	170	4.8	165	250	4.00	3.03	113.9	0.690
C - 11	1.35	37.9	171	4.2	167	227	4.41	3.26	119.9	0.718
C - 12	1.45	35.9	175	5.4	170	211	4.74	3.27	125.3	0.737
D - 1	1.37	13.5	63	3.6	59.4	227	4.40	3.21	42.6	0.717
D - 2	1.32	16.6	62	3.0	59.0	281	3.55	2.69	38.4	0.651
D - 3	1.13	11.7	64	3.3	60.7	193	5.19	4.59	46.2	0.761
D - 4	1.29	20.7	93	3.0	96.0	216	4.64	3.60	70.3	0.732
D - 5	1.36	24.3	99	3.3	95.7	254	3.93	2.89	65.4	0.684
D - 6	1.19	21.9	94	3.6	90.4	242	4.13	3.47	63.2	0.699
D - 7	1.27	27.4	129	3.9	125	219	4.56	3.59	91.0	0.728
D - 8	1.20	32.1	130	4.5	125	257	3.89	3.25	85.1	0.681
D - 9	1.25	30.3	129	4.2	125	242	4.13	3.30	87.4	0.699
D - 10	1.22	39.3	161	5.25	156	252	3.97	3.25	107.2	0.687
D - 11	1.22	37.5	163	4.35	159	236	4.24	3.48	112.4	0.707
D - 12	1.32	41.5	165	4.95	160	259	3.86	2.92	108.5	0.678

TABLE XXII
SUMMARY OF CHEMOSTAT RUNS E AND F

Chemostat Run No.	θ_c Cell Age days	X_1 Suspended Solids mg/l	$\text{PO}_4\text{-P}$							
			S_0	S_1	$S_0 - S_1$	$Y_n = \frac{X_1}{S_0 - S_1}$	$A = \frac{S_0 - S_1}{X_1}$	$q = \frac{S_0 - S_1}{X_1 \theta}$	S_e	f_e
			$\mu\text{g/l P}$	$\mu\text{g/l P}$	$\mu\text{g/l P}$	$\frac{\text{mg-cell}}{\text{mg P}}$	$\frac{\mu\text{g P}}{\text{mg-cell}}$	$\frac{\mu\text{g P}}{\text{mg-cell-day}}$	$\mu\text{g/l P}$	Fraction
E - 1	3.04	22.4	67.5	1.2	66.3	338	2.96	0.974	38.5	0.580
E - 2	2.87	24.7	68.7	1.2	67.5	366	2.73	0.952	36.8	0.545
E - 3	2.61	20.1	69.3	1.3	68	296	3.38	1.30	43.0	0.632
E - 4	3.06	32.2	104	1.05	103	314	3.20	1.05	63.0	0.612
E - 5	3.06	37.9	104	1.2	103	369	2.72	0.89	55.9	0.543
E - 6	2.69	36.1	100	1.35	98.6	366	2.73	1.02	53.6	0.544
E - 7	2.74	51.6	135	1.35	134	385	2.60	0.95	69.9	0.522
E - 8	2.69	54.2	136	1.5	134	404	2.47	0.92	67.0	0.500
E - 9	2.68	51.5	133	1.2	132	390	2.56	0.96	67.9	0.515
E - 10	2.62	66.3	166	1.65	164	404	2.47	0.94	81.5	0.497
E - 11	2.68	66.9	172	1.65	170	394	2.54	0.95	86.9	0.511
E - 12	2.86	61.5	169	1.8	167	380	2.72	0.95	90.5	0.542
F - 1	2.91	20.3	67.5	1.5	66.0	308	3.25	1.12	40.8	0.618
F - 2	2.86	20.0	68.7	1.35	67.3	297	3.37	1.18	42.5	0.631
F - 3	2.64	21.0	69.3	1.5	67.8	310	3.23	1.22	41.7	0.615
F - 4	2.87	29.6	104	1.2	103	288	3.48	1.22	66.2	0.643
F - 5	2.86	29.9	104	1.5	102	292	3.41	1.19	64.8	0.635
F - 6	2.74	28.8	100	1.5	98.5	291	3.42	1.25	62.6	0.636
F - 7	2.88	45.3	135	1.5	133	337	2.93	1.02	76.7	0.577
F - 8	2.68	42.0	136	1.65	134	313	3.19	1.19	81.9	0.611
F - 9	2.63	45.2	133	1.65	131	345	2.89	1.10	74.8	0.571
F - 10	2.61	58.2	166	1.8	164	355	2.81	1.08	91.7	0.559
F - 11	2.58	59.5	172	1.35	171	348	2.87	1.11	97.1	0.568
F - 12	2.82	57.1	169	1.65	167	338	2.92	1.04	96.0	0.575

TABLE XX:II

SUMMARY OF CHEMOSTAT RUNS G AND H

Chemostat Run No.	θ_c Cell Age days	X_1 Suspended Solids mg/l	PO ₄ -P							
			S_0	S_1	$S_0 - S_1$	$Y_n = \frac{X_1}{S_0 - S_1}$	$A = \frac{S_0 - S_1}{X_1}$	$q = \frac{S_0 - S_1}{X_1 \theta}$	S_e	f_e
			$\mu\text{g/l P}$	$\mu\text{g/l P}$	$\mu\text{g/l P}$	$\frac{\text{mg-cell}}{\text{mg P}}$	$\frac{\mu\text{g P}}{\text{mg-cell}}$	$\frac{\mu\text{g P}}{\text{mg-cell-day}}$	$\mu\text{g/l P}$	Fraction
G - 1	4.89	30.9	65.1	0.6	66.5	479	2.09	0.43	26.1	0.405
G - 2	4.69	32.7	69.0	0.45	68.5	477	2.09	0.45	27.9	0.407
G - 3	4.80	32.2	66.3	0.45	65.8	489	2.04	0.426	25.8	0.392
G - 4	4.82	55.4	103	0.45	102	543	1.84	0.382	33.2	0.325
G - 5	5.10	54.8	106	0.60	105	522	1.92	0.376	36.9	0.351
G - 6	5.08	55.8	101	0.9	100	558	1.79	0.353	30.6	0.306
G - 7	5.08	72.8	137	0.75	136	535	1.87	0.368	45.6	0.335
G - 8	4.58	75.4	137	0.6	136	554	1.80	0.394	42.8	0.315
G - 9	4.89	71.4	136	0.9	135	529	1.89	0.387	46.2	0.342
G - 10	4.78	91.5	172	0.75	171	535	1.87	0.391	57.5	0.336
G - 11	5.18	91.2	172	0.75	171	533	1.88	0.362	57.8	0.338
G - 12	5.51	94.7	171	0.9	170	557	1.79	0.326	52.4	0.308
H - 1	4.78	29.9	65.1	0.6	64.5	464	2.16	0.451	27.3	0.423
H - 2	4.78	31.3	69.0	0.45	68.5	457	2.19	0.458	29.6	0.432
H - 3	4.68	30.8	66.3	0.6	65.7	469	2.13	0.456	27.4	0.417
H - 4	4.82	51.8	103	0.75	102	508	1.97	0.409	37.5	0.368
H - 5	5.03	51.2	106	0.75	105	488	2.05	0.408	41.4	0.394
H - 6	4.97	49.1	101	0.75	100	491	2.04	0.410	39.0	0.390
H - 7	4.93	70.7	137	0.75	136	520	1.92	0.390	48.3	0.355
H - 8	4.55	69.5	137	0.75	136	511	1.96	0.430	49.6	0.365
H - 9	4.85	70.2	136	0.90	135	520	1.92	0.396	47.8	0.354
H - 10	4.62	89.0	172	0.9	171	520	1.92	0.416	60.4	0.353
H - 11	5.18	88.5	172	0.75	171	518	1.93	0.373	61.0	0.357
H - 12	5.42	87.7	171	0.9	170	516	1.94	0.357	60.9	0.358

TABLE XXIV
SUMMARY OF CHEMOSTAT RUNS I AND J

Chemostat Run No.	θ_c Cell Age days	$X_{\max}$ Suspended Solids mg/l	PO_4-P				
			S_0	S_1	$S_0 - S_1$	$Y_{\max} = \frac{X_{\max}}{S_0 - S_1}$	$A_0 = \frac{S_0 - S_1}{X_1}$
			$\mu g/l P$	$\mu g/l P$	$\mu g/l P$	$\frac{mg-cell}{mg P}$	$\frac{\mu g P}{mg-cell}$
I - 1	Nonflow System $\theta_c = \infty$ $Y_n = Y_{\max}$ $q = 0$ $S_e = 0$	49.6	65.1	0.15	64.9	764	1.31
I - 2		52.3	69.0	0.15	68.8	760	1.32
I - 3		48.0	66.3	0.3	66.0	727	1.38
I - 4		85.1	103	0.3	103	826	1.21
I - 5		84.2	106	0.3	106	794	1.26
I - 6		86.0	101	0.45	100	860	1.16
I - 7		120	137	0.45	136	883	1.13
I - 8		107	137	0.3	137	779	1.28
I - 9		111	136	0.45	135	824	1.22
I - 10		141	172	0.45	171	826	1.21
I - 11		131	172	0.45	171	768	1.31
I - 12		141	171	0.45	170	827	1.21
J - 1	Nonflow System $\theta_c = \infty$ $Y_n = Y_{\max}$ $q = 0$ $S_e = 0$	50.8	65.1	0.3	64.8	784	1.28
J - 2		54.7	69.0	0.3	68.7	796	1.26
J - 3		47.4	66.3	0.6	65.7	721	1.39
J - 4		84.2	103	0.3	103	817	1.22
J - 5		84.3	106	0.45	105	803	1.25
J - 6		85.0	101	0.45	100	850	1.18
J - 7		119	137	0.6	136	876	1.14
J - 8		111	137	0.45	136	816	1.23
J - 9		111	136	0.45	135	823	1.22
J - 10		130	172	0.6	171	760	1.32
J - 11		130	172	0.6	171	766	1.32
J - 12		131	171	0.6	170	772	1.30

Kinetic Analyses and Kinetic Constants. The growth characteristics of Selenastrum capricornutum were evaluated in terms of the following kinetic analyses, and kinetic constants were obtained with respect to a phosphorus-limiting condition:

- a. The net yield coefficient (Y_n) and its relation to cell age, and nutrient utilization constants, K_A , K_B .

$$Y_n = \frac{X_1}{S_0 - S_1} = Y_{\max} \left(1 - e^{-\theta_c / K_A} \right) \quad (39)$$

and

$$Y_n = \frac{X}{S_0 - S_1} = \frac{Y_{\max} \theta_c}{\theta_c + K_B} \quad (42)$$

- b. Nutrient removal velocity and nutrient utilization rate for cell growth.
- c. The maximum specific growth rate, $\hat{\mu}$, (day^{-1}).
- d. The decay rate, k_d , (day^{-1}).
- e. The half saturation constant, K_s , ($\mu\text{g P/l}$).

Net Yield Coefficient and Cell Continuity Equation. The net yield coefficients calculated from steady state cell concentrations and phosphorus uptake are listed in Tables XX to XXIV. The yield coefficients were significantly affected by the hydraulic residence time (or growth rate). The net yield increased with cell age up to a maximum cell yield coefficient ($Y_{\max}$). As discussed in the theory section (Section IV) this phenomenon appears to be caused primarily by the so-called "excess" nutrient uptake or more precisely "excess" phosphorus uptake. The cell continuity equations (Equation 20) modified to include a variable yield coefficient can be simplified and reduced to the following net yield coefficient-hydraulic residence time equations:

$$Y_n = Y_{\max} \left(1 - e^{-\theta_c / K_A} \right) \quad (39)$$

and

$$Y_n = Y_{\max} \left(\frac{\theta_c}{\theta_c + K_B} \right) \quad (42)$$

The maximum cell yield coefficient is defined as the cell yield coefficient at which there is no "excess" phosphorus uptake and it is determined experimentally in a nonflow cultural system (i. e., $\theta \rightarrow \infty$ days).

In a nonflow continuous culture system, the growth rate of the organism will approach the decay rate, i. e., $\mu = 1/\theta + k_d \rightarrow k_d$ when $\theta \rightarrow \infty$. The cell concentration will thus be maintained at a constant value where the cell matter produced per unit amount of nutrient utilized approaches the maximum yield coefficient. Thus:

$$Y_{\max} = \frac{X_{\max}}{S_0 - S_1}, \text{ when } \theta \rightarrow \infty, X_1 \rightarrow X_{\max}.$$

A plot of maximum steady state biomass ($X_{\max}$) against phosphorus uptake is shown in Figure 45. A maximum cell yield coefficient of 805 mg cell mass produced per mg of phosphorus removed was obtained with a correlation coefficient $r = 0.99$ ($p > 0.99$). This value corresponded to a cellular constant of 0.124% phosphorus.

By direct nonlinear and indirect linear transformation least square regression analyses of Equation 39 and 42, the best fit equations are (Figure 46):

$$Y_n = \frac{X_1}{S_0 - S_1} = 805 \left(1 - e^{-\frac{\theta_c}{4.78}} \right) \quad (68)$$

and

$$Y_n = \frac{X_1}{S_0 - S_1} = \frac{805 \theta_c}{\theta_c + 2.98} \quad (69)$$

in which $K_A = 4.78$ days and $K_B = 2.98$ days. Although both equations are highly significant ($p < 0.01$), the exponential equation (Equation 68) has a better correlation coefficient ($r = 0.99$) compared to $r = 0.98$ for the rectangular hyperbolic equation (Equation 69). The nutrient utilization constant, K_A , is estimated to be 4.78 days residence time which corresponded to a net yield, $Y_n = 0.632$ (maximum cell yield coefficient) (i. e., $Y_n = 0.632 Y_{\max}$) while the nutrient utilization constant K_B is estimated to be 2.98 days residence time which corresponds to a net yield, $Y_n = 0.5$ (maximum cell yield coefficient) (i. e., $Y_n = 0.5 Y_{\max}$).

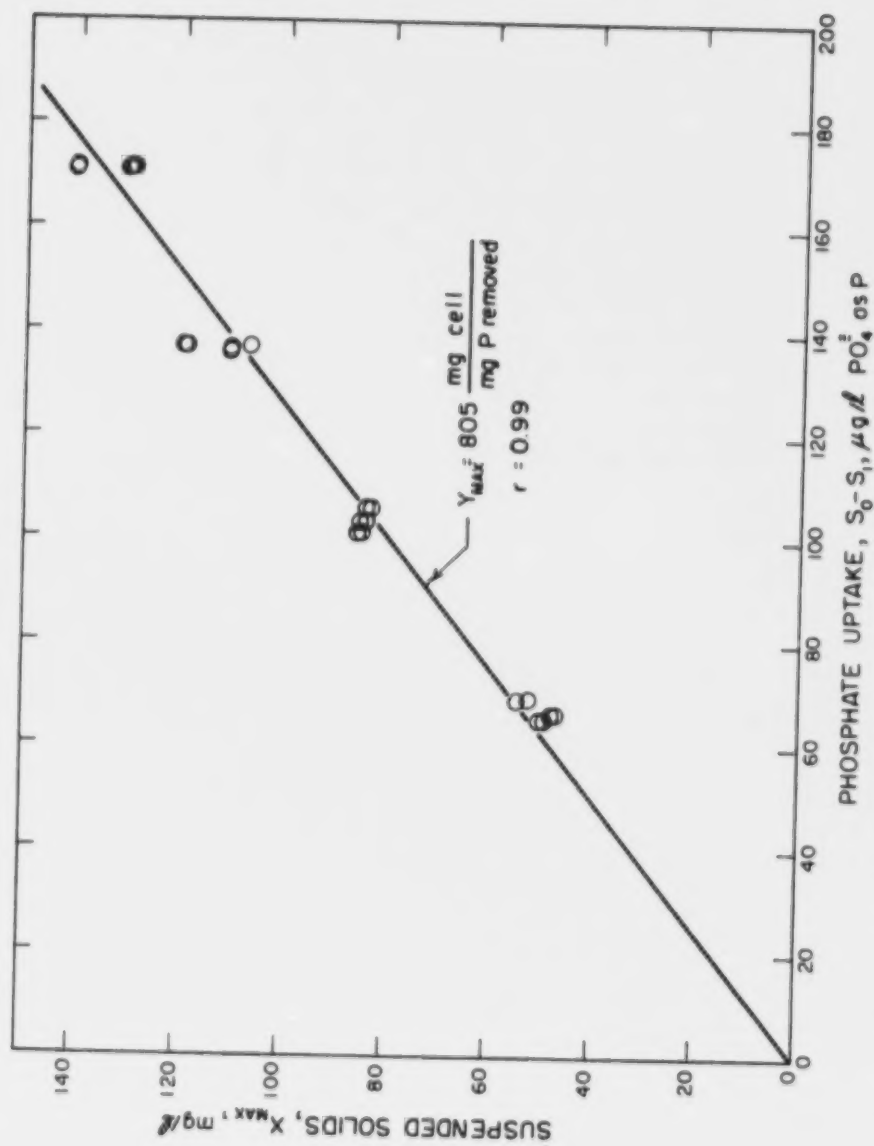


FIGURE 45. RELATIONSHIP BETWEEN STEADY STATE CELL CONCENTRATION AND PHOSPHATE UPTAKE AT INFINITE HYDRAULIC RESIDENCE TIME

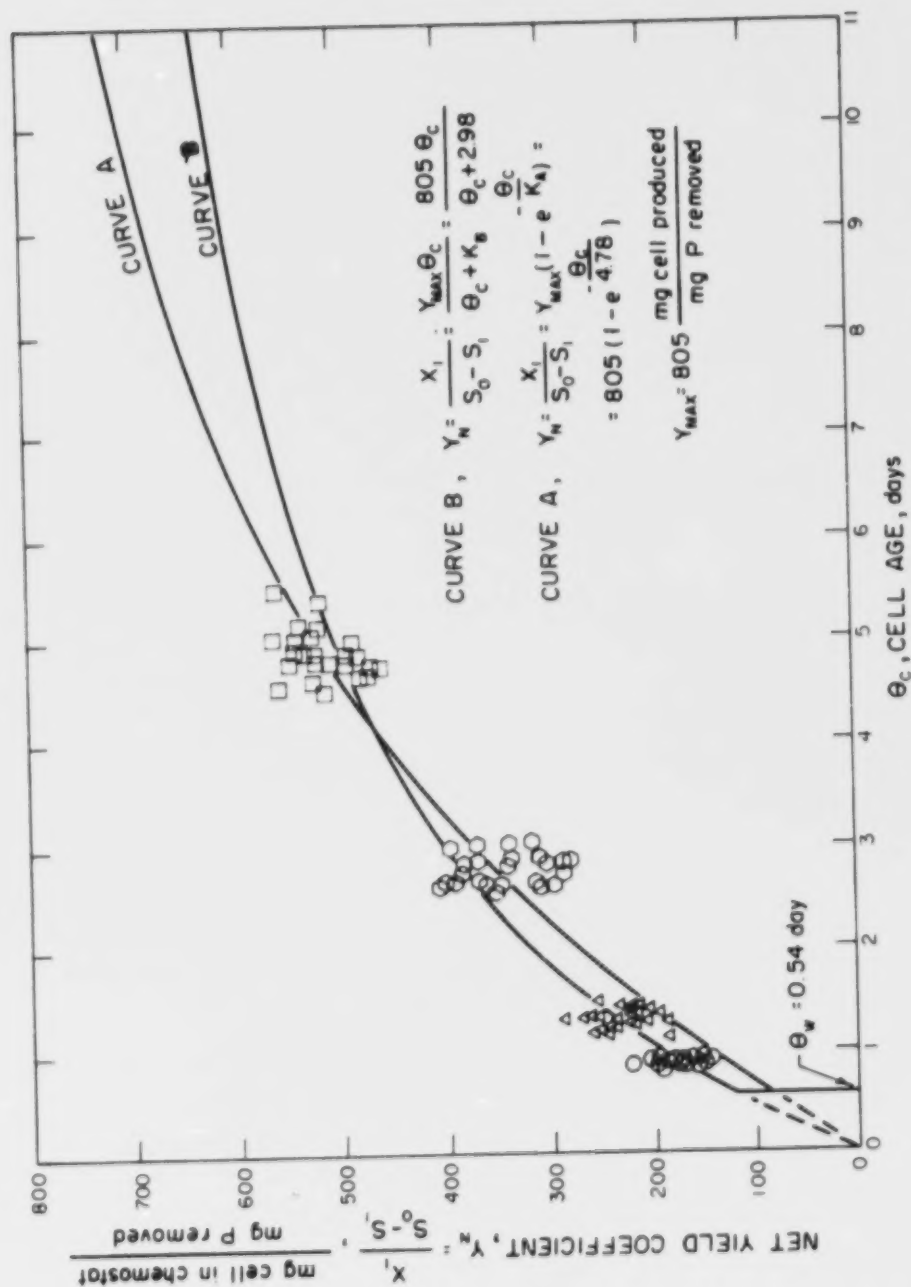


FIGURE 46. NET CELL YIELD COEFFICIENT AS A FUNCTION OF MEAN CELL AGE, θ_c

Nutrient Removal Velocity and Excess Phosphate Uptake.

As discussed in the theory section, when the nutrient removal velocity is greater than the rate of nutrient utilization for cell growth, the nutrient is accumulated and stored inside the cell in excess of the minimum nutrient requirement, or in other words "excess" nutrient uptake occurs.

The phosphorus removal velocities, q , at different cell ages are plotted against the specific growth rates, μ , in Figure 47. When no excess phosphorus uptake occurs, the equilibrium between the phosphorus removal velocity, q , and the phosphorus utilization rate, $\mu/Y_{\max}$, can be described by equation $q = \mu/Y_{\max}$ as shown by the dotted line in Figure 47. Consequently, no excess of phosphorus accumulates inside cells. On the other hand, the equation $q = \mu/(Y_n(1 + \theta_c k_d))$ indicates the inequality of the phosphorus uptake rate and utilization rate, and the effect of phosphorus accumulation and storage. The shaded area in Figure 47 represents the degree of excess phosphorus uptake rate with no excess phosphorus uptake at $\mu = k_d$, to a substantial excess phosphorus uptake at $\mu = \hat{\mu}$.

Amount of Excess Phosphorus Uptake and "Fraction of Excess Phosphorus Uptake".

A varying yield coefficient indicates a variable phosphorus content of the biomass at different hydraulic residence times. In this study the phosphorus content of the algal cells varied from 0.65% P at $\theta = 0.88$ day to 0.124% P at $\theta \rightarrow \infty$ days. The "excess" uptake of phosphorus by algae has been noted by several investigators [34, 96-98, 103, 104]. However, there is lack of knowledge as to the luxury phosphorus uptake mechanism and its kinetic characteristics. Based on the assumption that there is no excess phosphorus uptake at $\theta \rightarrow \infty$ days and any phosphorus content higher than 0.124% is considered to be in excess, Equations 45 and 46 were developed. Figure 48 shows the variation in the fraction of excess phosphorus uptake, $S_e/(S_0 - S_1)$, with hydraulic residence time.

$$f_e = e^{-\frac{\theta_c}{4.78}} \quad (r = 0.00, \quad p > 0.99) \quad (70)$$

or

$$f_e = \frac{\theta_c}{\theta_c + 2.98} \quad (r = 0.98, \quad p > 0.99) \quad (71)$$

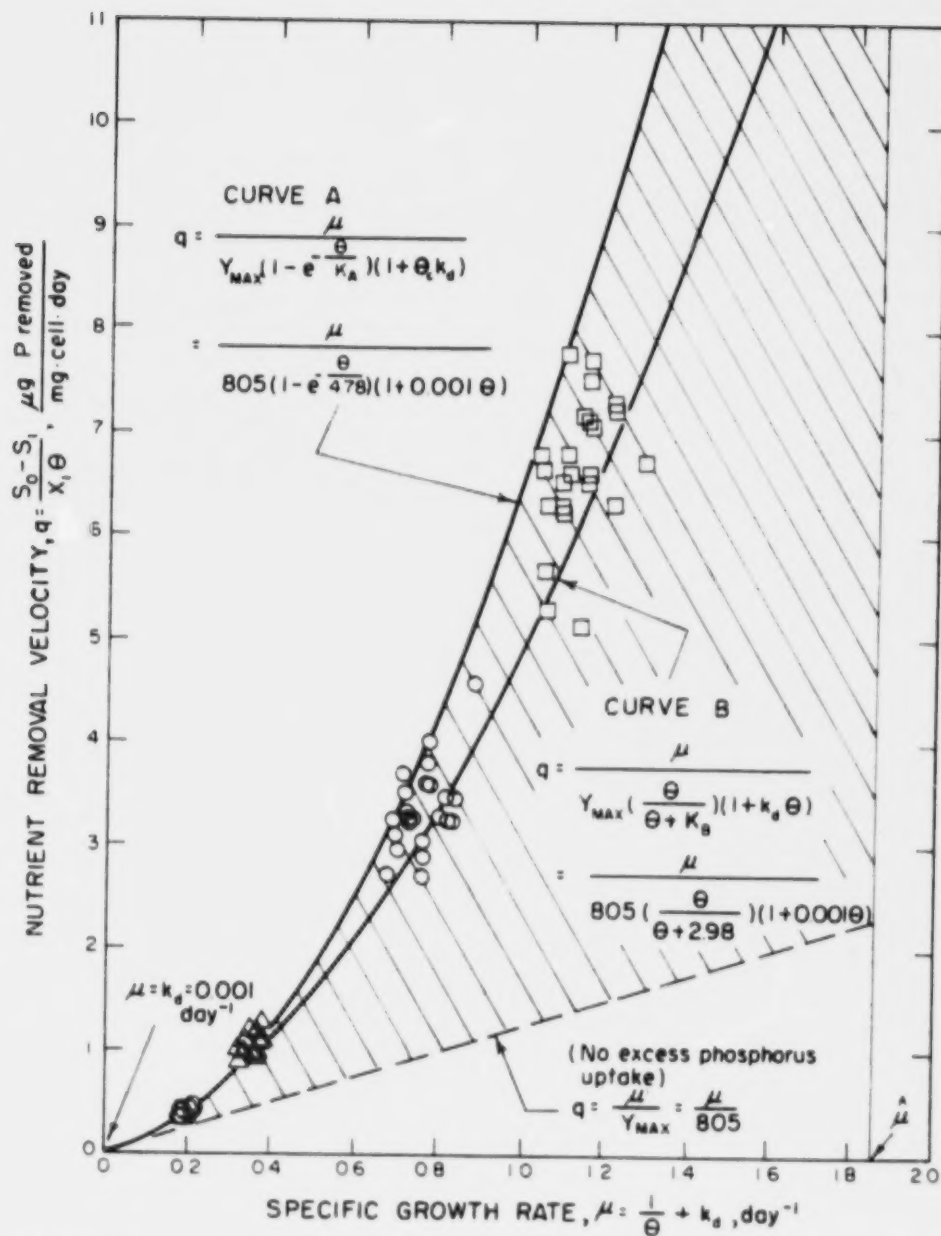


FIGURE 47. COMPARISON OF NUTRIENT REMOVAL VELOCITY, q , AND SPECIFIC GROWTH RATE, μ

Again according to the correlation coefficient, Equation 70 gives a better fit to the experimental data than Equation 71. Figure 48 indicates that the fraction of excess phosphorus uptake, $S_e/(S_0-S_1)$, is a simple function of mean cell age in the chemostat and it appears to be independent of input phosphorus concentration.

"Washout" Residence Time and Maximum Specific Growth Rate. In chemostat operation without cell recycle, the specific growth rate of algal cells is regulated by the hydraulic residence time ($\theta = \theta_c$) and is related to hydraulic residence time by,

$$\mu = \frac{1}{\theta} + k_d \quad (20)$$

where k_d is the decay constant. As residence time decreases, the specific growth rate increased until it reaches its maximum value ($\hat{\mu}$), and the residence time corresponds to its "washout," θ_w (Equation 30).

$$\hat{\mu} = \frac{1}{\theta_w} + k_d \quad (30)$$

The "washout" residence time was estimated experimentally to be 0.54 day and it corresponded to a maximum specific growth rate of $(1.85 + k_d) \text{ day}^{-1}$.

Evaluation of Michaelis-Menten, Monod Equation. The Michaelis-Menten, Monod (M-M, M) equation describing the growth rate limiting nutrient concentration can be used directly and indirectly to estimate the half saturation constant, K_s , the maximum specific growth rate, $\hat{\mu}$, and the decay rate constant, k_d . Substituting $(1/\theta_w + k_d)$ for $\hat{\mu}$ and $(1/\theta + k_d)$ for μ into the M-M, M equation, one obtains a modified M-M, M equation as follows:

$$\frac{1}{\theta} + k_d = \frac{\left(\frac{1}{\theta_w} + k_d\right) S_1}{K_s + S_1} \quad (72)$$

A direct nonlinear computer program (CAL G 2 NLIN Appendix B) was used to estimate the constants k_d and K_s . The nonlinear computer technique was used to minimize computational error with a linear transformation of the original nonlinear equation. However, a comparable indirect linear transformation regression analysis also was used to estimate the growth constants.

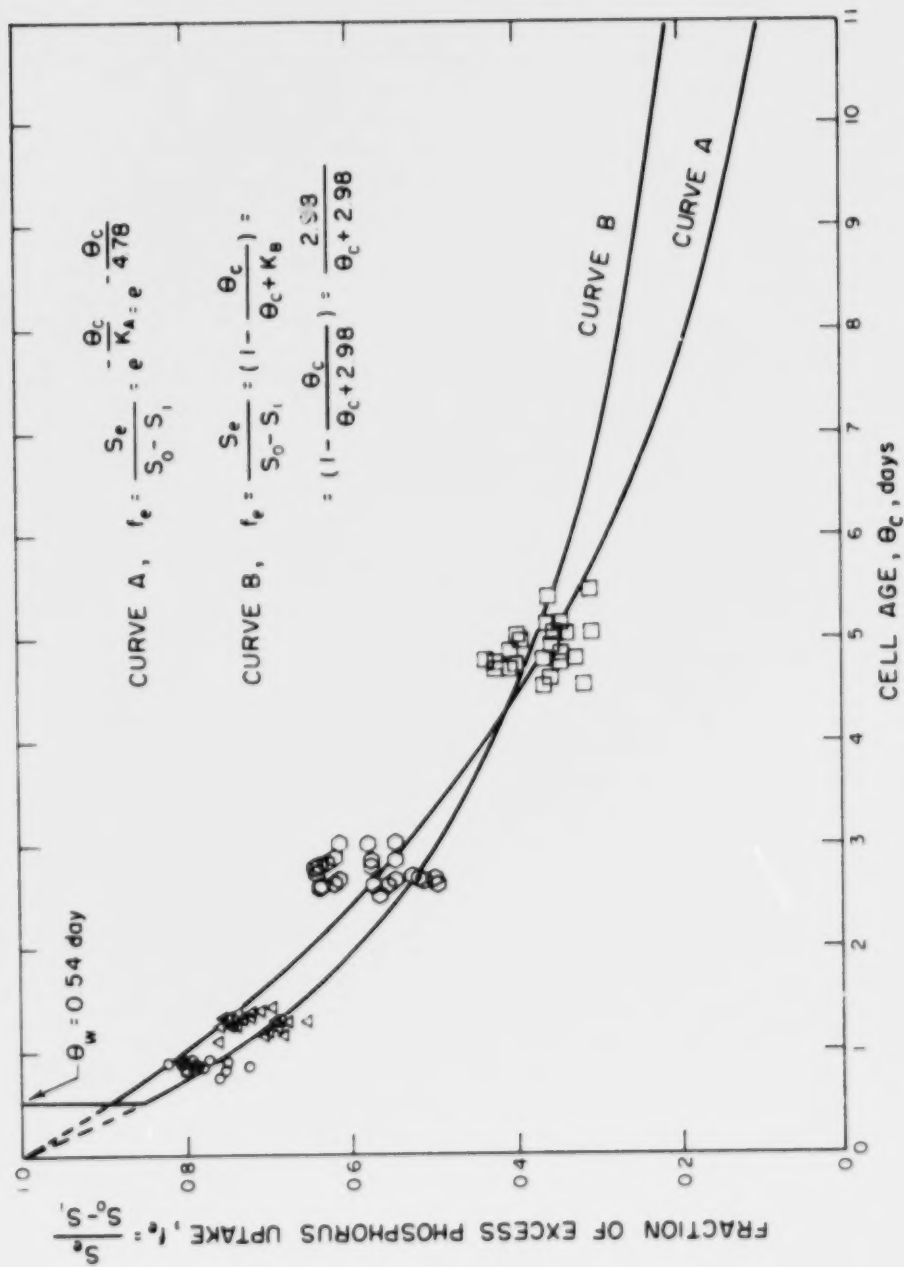


FIGURE 48. FRACTION OF "EXCESS" PHOSPHORUS UPTAKE AS FUNCTION OF MEAN CELL AGE, θ_c

Decay Constant, k_d . A decay rate of 0.001 per day was obtained directly from Equation 72 by nonlinear regression analyses. The small magnitude of k_d indicates that the rate of decrease in cellular mass due to endogenous respiration and cellular death with subsequent lysis is very small compared to the cell growth rate. Unfortunately many investigators neglect or ignore the decay rate because of its small magnitude. However, the decay rate which may be as large as 0.05 day^{-1} can play an important role in kinetic analyses. The small magnitude of the decay rate in this study (phosphorus-limiting condition) might be caused by the nutrient (phosphorus) "excess" uptake. Due to the high rate of nutrient uptake, the nutrient (phosphorus) released by cellular lysis may be taken up by cells immediately for cellular growth.

Maximum Specific Growth Rate. The maximum specific growth can be estimated from Equation 30 ($1/\theta_w = \hat{\mu} + k_d$) by knowing the "washout" residence time and decay constant. A maximum growth rate of 1.85 per day was obtained for *Selenastrum capricornutum* under the specific phosphorus-limiting condition. This value is in the same order of magnitude reported by the PAAP research group [34, 68]. This is no doubt but that the maximum specific growth should be greater than $(1/\theta_{\min} + k_d)$ where $\theta_{\min}$ is the minimum hydraulic residence time in which cells can maintain their steady state population without being washed out. In chemostat run #4, $\theta_{\min}$ was found to be 0.556 day which corresponds to a specific growth rate of 1.80 day^{-1} .

Half Saturation Constant, K_s . A half saturation constant of $5.67 \mu\text{g P/l}$ was obtained directly from Equation 72 by nonlinear regression analysis as shown by curve A in Figure 49. However, the best fit linear transformation ($S_1 = K_s \mu / (\hat{\mu} - \mu)$; S_1 as the dependent variable) gives a half saturation constant of $3.72 \mu\text{g P/l}$ with a correlation coefficient of 0.945 as shown by curve B in Figure 49. It is difficult to assign a single value for the saturation constant, K_s ; however, it appears that a range from 3.7 to $5.7 \mu\text{g P/l}$ best represents the most probable values for K_s of *Selenastrum capricornutum* with respect to a phosphorus-limiting condition. These low values for the half saturation constant for phosphorus imply that if phosphorus has been identified as the rate-limiting nutrient in Lake "A" and if the phosphorus concentration in Lake "A" is about $10 \mu\text{g P/l}$, a high rate of algal growth (Figure 49 shows a growth rate from 1.2 day^{-1} to 1.5 day^{-1} for *Selenastrum capricornutum* at a phosphorus concentration of $10 \mu\text{g P/l}$) likely exists in Lake "A."

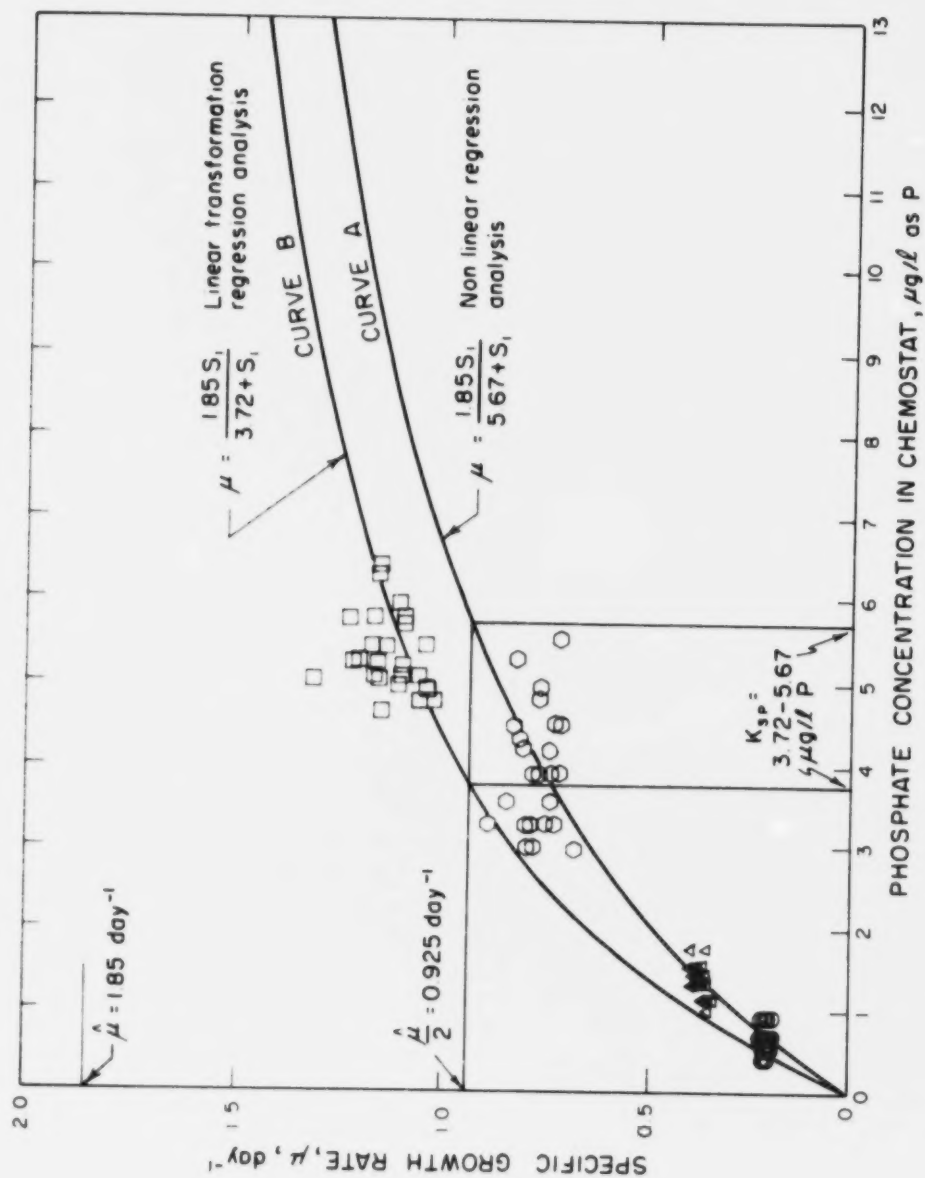


FIGURE 49. PLOT OF MICHAELIS-MENTEN (MONOD) EQUATION FOR PHOSPHORUS LIMITATION

Summary of Phosphorus Limitation Chemostat Studies

The most probable kinetic growth coefficients and constants developed from the data analysis for Selenastrum capricornutum under a phosphorus-limiting condition are summarized in Table XXV. Table XXV includes the coefficients and constants for all models evaluated and the results for each computational method. It is noted that all the regression equations in Table XXV are at a significance level of less than 1.0% (i. e. , $p \geq 0.99$).

Considering both the conceptual framework for the growth relationships as well as the level of statistical significance of the experimental data conforming to the kinetic models considered, the most probable models (best analytical formulations) and the best estimate of the kinetic constants and coefficients are summarized in Table XXVI.

It may be noted from Table XXVI that Equation 39 ($I_n = Y_{\max} (1 - e^{-\theta_c / K_A})$) best describes the relationship between net yield coefficient and cell age. The rectangular hyperbolic equation ($Y_n = Y_{\max} \theta_c / (K_B + \theta_c)$, Equation 42) can probably also be used to describe this net yield-cell age relationship but it does not appear to fit the experimental data as well as the exponential equation.

The half saturation constant, K_s , was found to be between 3.7 and 5.7 $\mu\text{g P/l}$ for Selenastrum capricornutum under phosphorus-limited conditions and it appears to be the most significant growth constant observed in this study. It should be noted that 3-6 $\mu\text{g P/l}$ for K_s is a very low level and its significance can be outlined with a simple practical example. Assume that in Lake "B" phosphorus is presumed to be the rate-limiting nutrient and serious consideration is being given to the partial, say 80%, removal of phosphorus from waste inputs which contribute presently about 50% of the total reactive phosphorus input to the lake.

Case 1. If the phosphorus concentration in Lake "B" is about 5 $\mu\text{g P/l}$ ($\approx K_s$ of phosphorus for Selenastrum capricornutum), it is at a level where the growth rate is significantly affected by the phosphorus concentration (approximately first order). Thus any reduction (say 80%) in the phosphorus content of the waste discharges would reduce proportionately the concentration of phosphorus in the lake ($\sim 40\%$) as well as the algal growth rate. Obviously the reduction in phosphorus concentration of the lake would only be affected by the fraction of the total phosphorus input removed by waste treatment. However, as the algal growth rate is almost linearly related to the phosphorus concentration in the lake, the maximum reduction of phosphorus possible would be beneficial from the standpoint of reducing the algal growth rate as well as the standing crop of algae.

TABLE XXV
KINETIC GROWTH MODELS AND CONSTANTS FOR *Selenastrum capricornutum*
UNDER PHOSPHORUS LIMITING CONDITIONS
(Various Models and Computational Methods)

Kinetic Model	Computational Method	Growth Kinetic Constants and Equations	Correlation Coefficient	Significance Level at:
$Y_n = \frac{X_1}{S_0 - S_1} = Y_{\max} \left(1 - e^{-\theta_c / K_A} \right)$ $\ln \frac{Y_{\max}}{Y_{\max} - Y_n} = \frac{\theta_c}{K_A}$	Linear Transformation Regression Analysis	$Y_n = 805 \left(1 - e^{-\theta_c / 4.78} \right)$ $Y_{\max} = 805 \frac{\text{mg cells produced}}{\text{mg P utilized}}$ $K_A = 4.78 \text{ days}$	0.99	<1%
$Y_n = Y_{\max} \left(\frac{\theta_c}{\theta_c + K_B} \right)$ $\frac{Y_n}{Y_{\max} - Y_n} = \frac{\theta_c}{K_B}$	Linear Transformation Regression Analysis	$Y_n = 805 \left(\frac{\theta_c}{\theta_c + 2.98} \right)$ $Y_{\max} = 805 \frac{\text{mg cells produced}}{\text{mg P utilized}}$ $K_B = 2.98 \text{ days}$	0.98	<1%
$\mu = \frac{\hat{\mu} S_1}{K_s + S_1}$ $\frac{1}{\hat{\mu} + k_d} = \frac{\left(\frac{1}{\hat{\mu}} + \frac{1}{k_d} \right) S_1}{K_s + S_1}$	Nonlinear Regression Analysis	$\mu = \frac{1.85 S_1}{5.67 + S_1}$ $k_d = 0.001 \text{ day}^{-1}$ $\hat{\mu} = 0.54 \text{ day}^{-1}$ $\hat{\mu} = 1.85 \text{ day}^{-1}$ $K_s = 5.67 \mu\text{g P/l}$		<1%
$S_1 = \frac{K_s \mu}{\hat{\mu} - \mu}$	Linear Transformation Regression Analysis	$\mu = \frac{1.85 S_1}{3.72 + S_1}$ $\hat{\mu} = 0.54 \text{ day}^{-1}$ $\hat{\mu} = 1.85 \text{ day}^{-1}$ $K_s = 3.72 \mu\text{g P/l}$	0.95	<1%
$\mu = \hat{\mu} \left(1 - e^{-S_1 / K_A} \right)$ $S_1 = K_A \ln \frac{\hat{\mu}}{\hat{\mu} - \mu}$	Linear Transformation Regression Analysis	$\mu = 1.85 \left(1 - e^{-S_1 / 4.06} \right)$ $\hat{\mu} = 1.85 \text{ day}^{-1}$ $K_A = 4.06 \mu\text{g P/l}$ $K_s = 4.19 \mu\text{g P/l}$	0.98	<1%
$\mu = \frac{\hat{\mu} S_1^n}{K_s + S_1^n}$	Nonlinear Regression Analysis	$\mu = \frac{1.85 S_1^{1.07}}{5.83 + S_1^{1.07}}$ $\hat{\mu} = 1.85 \text{ day}^{-1}$ $n = 1.07$ $K_s = 5.83 \mu\text{g P/l}$		<1%

TABLE XXVI
THE MOST PROBABLE KINETIC GROWTH MODELS AND BEST
ESTIMATE OF GROWTH CONSTANTS AND COEFFICIENTS
FOR Selenastrum capricornutum UNDER
PHOSPHORUS LIMITED CONDITIONS

Kinetic Growth Relationship	Most Probable Equation and Best Estimate of Coefficient
Net yield coefficient - cell age relationship (Modified Cell Continuity Equation)	$Y_n = \frac{X_1}{S_0 - S_1} = Y_{\max} \left(1 - e^{-\theta_c / K_A} \right)$ $Y_{\max} = 805 \frac{\text{mg cells produced}}{\text{mg P utilized}}$ $K_A = 4.78 \text{ day}$
Fraction of "excess" phosphorus uptake - cell age relationship	$f_e = e^{-\theta_c / K_A}$ $K_A = 4.78 \text{ day}$
Specific growth rate - rate limiting nutrient concentration relationship	Michaelis-Menten, Monod Equation $\mu = \frac{\mu S_1}{S_1 + K_s}$ $k_d = 0.001 \text{ day}^{-1}$ $\hat{\mu} = 1.85 \text{ day}^{-1}$ $K_s = 3.7 \sim 5.7 \mu\text{g P/l}$

Case 2. If the phosphorus content in Lake "B" is about $50 \mu\text{g P/l}$ ($\approx 10 \times K_s$), the specific growth rate of Selenastrum capricornutum is near its maximum value (i. e., $\mu \rightarrow 1.85 \text{ day}^{-1}$). Any increase in the phosphorus concentration of the lake would have no measureable effect on the maximum growth rate nor on the standing crop. Correspondingly, a reduction of say 80% of the waste discharge phosphorus would only reduce the total phosphorus input by 40% and the phosphorus concentration in the lake would still be about $30 \mu\text{g P/l}$. At this level of phosphorus concentration (i. e., $\sim 6 \times K_s$), the specific growth rate is still very near its maximum level (i. e., $\mu \sim 1.80 \text{ day}^{-1}$) and correspondingly there would be no measureable decrease in the standing crop of algae. The expenditure for phosphorus removal would be essentially wasted. It would have been far more effective if a part of the money spent for phosphorus removal had been spent on determining the growth rate limiting nutrient or factors. It is obvious that if the phosphorus content is as high as $50 \mu\text{g P/l}$, it is very unlikely (if not impossible) that phosphorus is rate-limiting for an organism with the growth characteristics of Selenastrum capricornutum. Once the growth-limiting nutrient or factor is identified, attention can be directed to the economic and technical feasibility of its removal and the likely effects on the growth rate and standing crop of algae.

Chemostat Study on Natural Water (Sacramento River)

Chemostat algal bioassays were conducted on samples of the Sacramento River (California) to determine: 1) the linearity of steady state cell concentration response, 2) the identification of the growth-limiting nutrient, and 3) the interpretation and application of kinetic growth constants from natural water chemostat bioassays.

Water samples from the Sacramento River were pretreated by autoclaving. Twenty-liter aliquots of 100%, 66.7%, and 33.3% (V/V) were produced by diluting Sacramento River waters with autoclaved distilled deionized water. Twelve chemostats (3-30% PAAP control, 3-100% Sacramento River waters, 3-66.6% Sacramento River water and 3-33.3% Sacramento River samples) were operated at a hydraulic residence time of about 2.6 days. Cell counts by hemocytometer and pH measurements were performed daily, while nutrient analyses (NH_4^+-N , $(\text{NO}_2 + \text{NO}_3)-\text{N}$, $\text{PO}_4^{3-}-\text{P}$), cell counts, and pH were performed at steady state conditions. Table XXVII summarizes all the daily and steady state measurements for each parameter.

The Linearity of Steady State Cell Concentration Response. Steady state cell concentration based on cell counts and suspended solids were determined and related to the dilutions of Sacramento River water as shown in Figure 50. A highly significant ($p \geq 0.99$)

TABLE XXVII
SUMMARY OF CHEMICAL ANALYSIS RESULTS ON SACRAMENTO RIVER WATER

Chemostat Number	Type of Sample	Day of Operation												Hydraulic Residence Time, θ (days)			
		5			6			7			8						
		pH	OD ^a or Cell Counts	pH	OD or Cell Counts	pH	OD or Cell Counts	pH	OD or Cell Counts	Suspended Solids mg/L	10 ₀ -P		NH ₄ -N		NO ₃ -N		
											S ₀ mg P/l	S ₁ mg P/l	S ₀ mg N/l		S ₁ mg N/l	S ₀ mg N/l	S ₁ mg N/l
1	Control 10% FAXP	8.0	0.285	8.2	0.200	8.3	0.230	8.3	0.225	76.0	186	1.9	0	4200	428	2.85	
2		7.8	0.285	8.2	0.200	8.3	0.220	8.4	0.198	66.0	186	1.2	0	4200	579	2.35	
3		7.3	0.220	7.3	0.200	8.2	0.200	8.3	0.190	63.5	186	2.1	0	4200	554	2.30	
4	100% Sacramento River Water	7.9	223	8.0	146	8.3	162	8.2	147	18.7	34.2	2.7	25.2	107.6	9.8	2.85	
5		8.1	167	7.8	125	8.4	154	8.0	150	21.9	34.2	3.9	25.2	107.6	11.1	2.94	
6		8.0	154	7.6	139	8.3	114	7.7	145	20.2	34.2	4.2	25.2	107.6	8.2	2.85	
7	66.7% Sacramento River Water	8.1	130	8.0	106	8.1	124	7.8	112	8.3	22.1	3.3	16.6	75.0	9.3	2.56	
8		8.1	144	7.7	100	8.0	103	8.1	102	7.1	22.1	4.5	16.6	75.0	6.5	2.56	
9		7.8	143	8.3	85	8.0	98	7.9	93	6.1	22.1	4.8	16.6	75.0	6.5	2.41	
10	11.3% Sacramento River Water	7.9	37	8.4	35	8.2	52	8.0	48	4.1	3.7	4.6	14.3	36.7	8.3	2.63	
11		8.1	90	8.5	76	8.3	85	7.9	70	5.1	3.7	4.8	14.3	36.7	9.5	2.60	
12		8.2	59	8.3	49	8.4	40	7.9	43	3.0	3.7	4.8	14.3	36.7	11.3	2.56	

^aOD = absorbance at 600 mμ.

^bCell counts = cell number $\times 10^4$ /ml (microtometer).

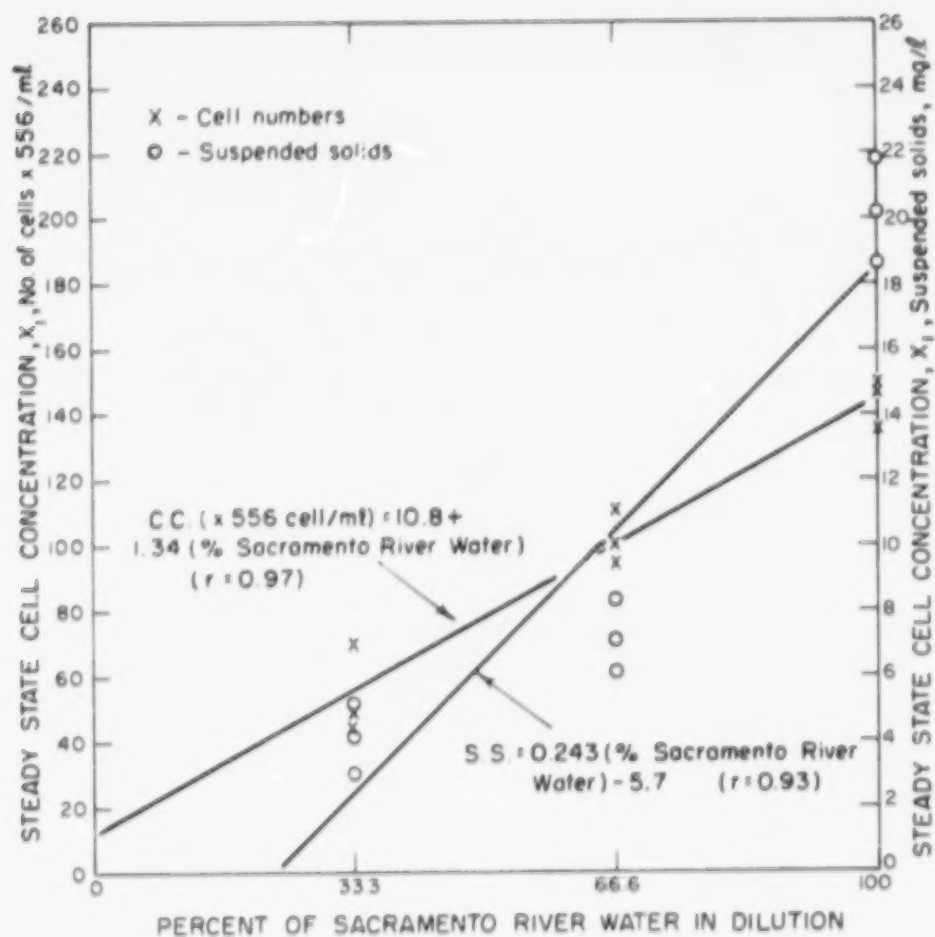


FIGURE 50. RELATIONSHIP BETWEEN STEADY STATE CELL CONCENTRATION AND DILUTION OF SACRAMENTO RIVER WATER

correlation coefficient ($r = 0.97$, $n = 9$) was observed between the steady state cell counts (hemocytometer) and the dilutions of Sacramento River water.

$$\text{Cell counts (x 556 cells/ml)} = 10.8 + 1.34 (\% \text{ Sacramento River Water}). \quad (73)$$

On the other hand, although a highly significant ($p > 0.99$) correlation coefficient ($r = 0.93$, $n = 9$) was obtained between the steady state suspended solids and the dilutions of Sacramento River water, this relationship shown in Figure 50 and described below,

$$\text{Suspended solids (mg/l)} = 0.243 (\% \text{ Sacramento River Water}) - 5.7 \quad (74)$$

was unsatisfactory due to the large scatter in the data. The large scatter in the data was likely due to the fact that the nutrient level in diluted Sacramento River samples could not support enough algal growth to produce a high enough level of X_1 (suspended solids ≈ 4 mg/l @ 33%) to insure adequate precision with the suspended solids determination. One of the problems with the use of suspended solids as a cell concentration parameter is inadequate precision at low biomass levels. Also, there was considerable suspended particulates in the Sacramento River water which interfered with the use of a suspended solids measurement as a cell concentration parameter.

The linearity of steady state cell concentration (hemocytometer cell counts) and the dilution of natural water indicates that there is no evidence of toxicants present in Sacramento River water. It is readily apparent that the chemostat can be used efficiently for assaying for relative algal response for waters of moderate nutrient content as well as for waters rich in nutrients.

Algal Growth Kinetic Characteristics. Table XXVIII summarizes the observed parameters of cell concentration, soluble orthophosphate and nitrate-nitrite-ammonia nitrogen for the presumed steady state conditions for the chemostat assay of Sacramento River water. The cell concentration expressed in terms of suspended solids was estimated from the relationships between cell counts, absorbance, and suspended solids reported by Porcella *et al.* [34].

Net Yield Coefficient $X_1/(S_0 - S_1)$. The net yield coefficients were calculated based on phosphate and total soluble nitrogen ($\text{NO}_3^- + \text{NO}_2^- + \text{NH}_4^+ - \text{N}$). A high variation of net yield coefficient for phosphate was observed, the values ranging from 417 to 1310 mg

TABLE XXVIII
 PUTNIMED STEADY STATE CHEMOSTAT PARAMETERS FOR SACRAMENTO RIVER WATER

Chemostat Number	Type of Sample	X_0 Cell Biomass Suspended Solids ^a mg/l	μ Residence Time (days)	μ Specific Growth Rate day ⁻¹	100μ P			$NO_3 + NO_2 + NH_4-N$				
					y_{N_2} mg P/l	S_N mg P/l	Net Yield $\frac{X_A - X_0}{S_0 - S_A}$ mg cell / mg N	Calculated y_{N_2} mg P/l	S_0 mg N/l	S_A mg N/l	Net Yield $\frac{X_A - X_0}{S_0 - S_A}$ mg cell / mg N	Calculated y_{N_2} mg N/l
1	Control 10% FAAP	85.7	2.89	0.331	186.0	1.5	454	6.4	4200	628	24.0	26.0
2		74.2	2.35	0.426	186.0	4.2	401	4.0	4200	579	20.5	19.5
3		72.3	2.30	0.436	186.0	2.4	393	6.8	4200	554	19.8	18.0
4	100% Sacramento River Water	13.0	2.85	0.331	34.2	2.7	429	11.3	133.0	9.8	11.0	11.8
5		13.8	2.94	0.344	34.2	5.9	433	17.2	133.0	15.2	11.5	98.4
6		12.5	2.85	0.351	34.2	4.2	417	13.0	133.0	8.2	10.0	15.0
7	66.7% Sacramento River Water	10.3	2.86	0.334	22.4	3.3	548	12.3	91.6	9.7	12.6	56.2
8		9.4	2.86	0.331	22.4	4.3	533	16.8	91.6	6.5	11.0	24.3
9		8.8	2.41	0.416	22.4	4.8	508	16.8	91.6	6.5	10.3	22.4
10	33.3% Sacramento River Water	4.4	2.63	0.384	5.7	4.8	898	18.3	31.0	9.3	10.6	35.8
11		4.4	2.60	0.383	5.7	4.8	1310	18.3	31.0	9.3	15.4	36.2
12		3.7	2.56	0.391	5.7	4.8	857	17.9	31.0	12.1	10.8	45.2

^a Estimated from the biomass parameter relationships of Porcella et al. (34).

$b_0 = \frac{1}{\theta} + y_{N_2}$ and $b_0 = 0.004 \text{ day}^{-1}$.

$y_{N_2} = \frac{S_0 - S_A}{X_A - X_0}$ where $S_0 = 1.54 \text{ day}^{-1}$.

cells produced per mg $\text{PO}_4\text{-P}$ removed. This variation was probably caused by a combination of factors such as the low precision of the phosphate determination at low concentration, the poor precision of biomass determination (cell counts) at low cell levels, and the possibility of particulate matter supplying phosphorus for cell growth. These values are markedly higher than those estimated from the phosphate-limiting kinetic study (Y_n for phosphate at a $\theta = 2.6$ days was about 350 mg cells produced per mg $\text{PO}_4\text{-P}$ removed). It is apparent that the data on phosphorus yield coefficients does not provide any indication that phosphorus was the growth rate limiting nutrient.

The net yield coefficients based on nitrogen were relatively constant ranging from 10.0 to 15.4 with an average of 11.5 mg cells produced per mg nitrogen removed. These values are significantly lower than the corresponding values reported by Porcella et al. [34] (Y_n for nitrogen was about 20 ~ 30 mg cells produced per mg nitrogen removed). It appears that nitrogen was not the growth rate limiting nutrient. It should be noted that the possibility existed for the hydrolysis of nitrogenous compounds in particulate matter present in the samples which would tend to increase the observed yield coefficients.

Half Saturation Constants, K_s . Using the kinetic data for Selenastrum capricornutum obtained from the phosphate-limiting algal studies (maximum specific growth rate, μ , and decay constant k_d), the half saturation constants for phosphate and nitrogen were calculated and are presented in Table XXVIII

Nitrogen, K_s . An average half saturation constant of 2140 $\mu\text{g N/l}$ and 37.3 $\mu\text{g N/l}$ was obtained for the 30% PAAP control assay and Sacramento River assays. Unfortunately, there are no reliable kinetic data for Selenastrum capricornutum with respect to nitrogen. It is obvious that nitrogen was not a growth-limiting nutrient in the 30% PAAP control. However, based on the K_s data there was a possibility that nitrogen was the growth rate limiting nutrient in Sacramento River samples.

Phosphorus, K_s . With respect to phosphorus, average half saturation constants of 5.7 $\mu\text{g P/l}$ and 16.9 $\mu\text{g P/l}$ were observed for the 30% PAAP control and Sacramento River samples, respectively. Comparing these values with the half saturation constant obtained from the phosphorus-limiting algal growth kinetic study (K_s for phosphorus 3.7 to 5.8 $\mu\text{g P/l}$), it is apparent that in the 30% PAAP medium, phosphorus is the primary growth-limiting nutrient. On the contrary, the high value observed for the K_s of Sacramento River water indicates that phosphorus definitely is not the growth rate limiting nutrient.

The foregoing analyses indicate the very great utility in the application of kinetic constant data in identifying the growth-limiting nutrient in natural water samples. However to do so, one must have reliable kinetic data for the nutrient and organism of concern. Based upon the data obtained from a single series of chemostat assays of a natural water, the observed data on the half saturation constant K_s and the yield coefficient, Y , permits a first order assessment of the likely growth rate limiting nutrients.

Enrichment (Spiking) Studies. To identify the growth-limiting nutrient by enrichment (spiking) procedures, 3 undiluted Sacramento River chemostats were operated in accordance with the spiking procedure outlined in Table XXIX. Daily cell counts and pH observations were made and these data are also reported in Table XXIX. Figure 51 shows the variation in cell counts as they responded to the different spiking treatments. In examining the changes of cell counts with time for the different spiking treatments, in Figure 51 one can conclude that there were no significant effects on cell counts with spiking treatment A, B, C, and D. In other words, the cell concentration of the chemostats were not affected by adding macronutrients (Mg, Ca, K, S, Cl), iron, phosphate, or nitrogen. However, the cell concentrations increased about 4 times in about 27 hours after spiking with macronutrients + Fe + P + N + micronutrients. This indicates that macronutrients, Fe, P, or N were not the growth-limiting nutrient in Sacramento River water. However, when micronutrients were added (plus all others) growth increased markedly, indicating that one of the micronutrients was limiting the growth rate. Unfortunately, the specific growth-limiting micronutrient could not be identified because all micronutrients were lumped together as specified in the standard PAAP procedure [35]. Unfortunately, the spiking treatment included all of the macronutrients, including N and P as well as the micronutrients. It would have been better and the results would have been more definitive if a spiking treatment of only micronutrients had been used. Similarly it would be possible to determine which of the micronutrients was rate-limiting by individual spiking treatments of each micronutrient. Unfortunately time and funds did not permit such studies.

It should be noted that the batch assay spiking studies did not identify with certainty the rate-limiting nutrient. However, the chemostat spiking studies of the same sample indicated clearly the rate-limiting nutrient. This is additional evidence of the general applicability of the chemostat in identifying the rate-limiting nutrients, as well as for determination of the kinetic characteristics of each organism and nutrient. The application of kinetic information to practical problems has been described in detail and it appears to be the most promising method for assessing the general eutrophication problem.

TABLE XXIX
CHEMOSTAT ENRICHMENT (SPIKING) PROCEDURE AND OBSERVED
DATA WITH SACRAMENTO RIVER WATER

Day of Operation	Enrichment (Spiking) With ^a	100% Sacramento River Water Chemostats No.						Control Chemostat ^c	
		1		2		3			
		pH	Cell ^b Count	pH	Cell Count	pH	Cell Count	pH	OD ^d
8	Macronutrients ^e	8.2	147	8.0	150	7.7	135	8.3	0.225
9	Macronutrients	8.3	176	8.0	179	7.9	171	8.4	0.230
10	Macronutrients + Fe	7.8	189	7.7	184	7.9	170	7.8	0.200
11	Macronutrients + Fe	7.8	220	8.2	213	8.2	196	7.7	0.190
12	Macronutrients + Fe + P	8.1	182	8.3	183	8.3	168	7.6	0.190
13	Macronutrients + Fe + P	8.2	173	8.3	161	8.3	150	7.5	0.195
14	Macronutrients + Fe + P + N	8.3	185	8.2	176	8.3	160	7.8	0.190
15	Macronutrients + Fe + P + N	8.4	211	8.3	189	8.4	178	8.4	0.190
16	Macronutrients + Fe + P + N + micronutrients	8.3	206	8.3	195	8.4	170	8.3	0.200
17	Macronutrients + Fe + P + N + micronutrients ^f	8.4	758	8.3	800	8.4	863	8.4	0.205

^aFinal concentration of spiked nutrients = 30% PAAP medium.

^bCell number x 556/m³ (hemacytometer) at time of enrichment.

^cControl chemostat operated on 30% PAAP medium; received no enrichment.

^dOD = absorbance at 600 mμ.

^eMacronutrients including Mg, Ca, K, S, and Cl.

^fMicronutrients spike identical in composition and concentration to that of 30% PAAP medium.

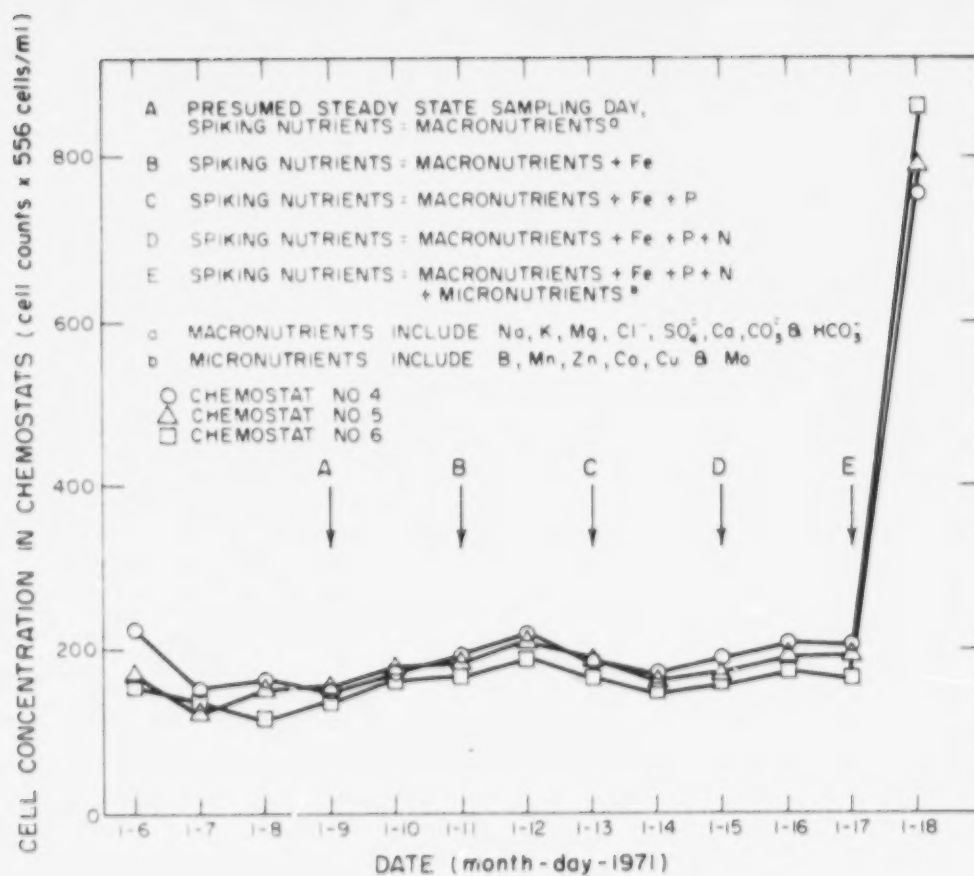


FIGURE 51. SACRAMENTO RIVER CHEMOSTAT CELL CONCENTRATION, X_1 , VARIATION WITH DIFFERENT SPIKING TREATMENTS

SECTION VII

COMPARISON OF BATCH AND CHEMOSTAT ALGAL ASSAYS

To define the role of each of the batch and chemostat assays in eutrophication analyses it is necessary to compare the assays on the basis of the defined requirements of algal assays.

GROWTH RESPONSES

Proportionality of Cell Concentration to Concentration of Growth-Limiting Nutrient

Synthetic Media The cell concentration parameters $\bar{X}$ for the batch assay and X_1 for the chemostat assay were found to be significantly related ($p = 0.99$) to the concentration of the growth-limiting nutrient. However, it was also shown that in batch assays the yield coefficient (Y) for nitrogen and phosphorus were affected by the initial N/P ratios in the cultures and in chemostats Y was not constant but a function of the residence time. Therefore, the linearity of cell concentration responses is affected by culture conditions and these conditions should not be ignored.

Natural Water Samples The cell concentration responses $\bar{X}$ and X_1 were linearly related ($p = 0.99$) with the dilution of the sample, i.e., the more concentrated the sample the greater the cell density. In both methods this linearity can be used to detect the presence of toxicants if growth rate is the parameter used, however, in the batch assay small reductions in the growth rate are not detected while such effects can be observed easily with chemostat assays. The chemostat is much more sensitive than the batch assay for the detection of toxic effects.

In general, the basic requirement that algal growth and the resulting cell concentration should be proportional to the concentration of the growth-limiting nutrient is fulfilled with both the batch and chemostat assay method. However, this has been shown to hold only for samples or media with relatively high concentrations of nutrients ($P \geq 30 \mu\text{g}/\ell$ and $\text{NH}_3\text{-N} + \text{NO}_3\text{-N} \geq 125 \mu\text{g}/\ell$).

Relationship of Growth Rate With Concentration of Growth-Limiting Nutrient The growth rate parameters, μ_b for the batch assay and μ for the chemostat assay were both related to the concentration of the growth-limiting nutrient with the first order-zero order type of relationship (i.e., Michaelis-Menten, Monod equation). However, the

relationship only can be estimated crudely in batch assays apparently due to the transient character of the cell concentration and nutrient concentrations, whereas the chemostat assays with constant cell concentrations and nutrient concentrations permit rather precise determination of the above relationship. The chemostat method is far superior to the batch method in determining growth rate-growth limiting nutrient relationships.

Precision of Growth Responses. The algal growth responses $\hat{X}$ and $\hat{\mu}_b$ of the batch assay can be determined with reasonable accuracy (coefficient of variation approximately 15%). However, the chemostat growth response X_1 can be determined with a higher precision (coefficient of variation 10%). The precision of the chemostat assay appears to be significantly higher than that of batch assays.

IDENTIFICATION OF THE GROWTH-LIMITING NUTRIENT

Yield Coefficients

In batch cultures the use of yield coefficients (Y) proved to be highly variable and of virtually no value in determining which nutrient limited growth. In chemostats Y_P was less variable than with batch assays but it was found to be a function of the residence time (growth rate) and thus was limited in its use in identifying the growth-limiting nutrients. However, once the relationship between each nutrient yield coefficient and residence time is determined (as was done for P in this study) the use of yield coefficients in chemostat studies may be of substantial value.

Enrichment (Spiking) Techniques

The value of spiking techniques in batch cultures to identify the growth-limiting nutrient was variable because the $\hat{X}$ response was not sufficiently sensitive to identify clearly significant differences in spiking. Only the $\hat{\mu}_b$ response was able to demonstrate clearly that a micro-nutrient was limiting growth in a specific water. $\hat{\mu}_b$ appears to be the most sensitive growth parameter for identifying the growth-limiting nutrient in batch assays by spiking treatments. Moreover, the use of $\hat{\mu}_b$ permits direct interpretations or predictions based upon conceptual eutrophication models such as presented in Figure 1 whereas the relationship between $\hat{X}$ and the conceptual model is not at all defined, that is no theory or empirical knowledge exists on the relation between $\hat{X}$ and the cell concentration in natural waters.

Spiking treatments with the chemostats (at a constant residence time) clearly identified the growth-limiting nutrient in a natural water sample

(Sacramento River). Addition of other nutrients caused no significant increase in X_1 , but addition of the growth-limiting nutrient (micro-nutrients) caused an immediate response in the cell concentration, X_1 , indicating that growth was limited specifically by a micronutrient. Unlike the batch assays, the chemostat results identified unequivocally which nutrient limited growth. The chemostat assay appears to be much more sensitive than the batch assay to spiking treatments in the identification of the growth-limiting nutrients.

Half Saturation Constants

The batch assay method is not adequate for the accurate determination of kinetic constants especially the half saturation constants (K_s). This is a severe limitation because of both the significance and role of K_s in identifying the growth-limiting nutrients. The chemostat is ideally suited for determination of K_s values for specific organisms and nutrients. Comparison of K_s values obtained with samples for presumed rate-limiting nutrients and with the true K_s values determined for the organism and the actual rate-limiting nutrient permits identification of the growth-limiting nutrient. This was demonstrated for both the PAAP medium as well as natural water samples. The ability to use chemostat-determined K_s values to identify growth-limiting nutrients indicates the versatility of the chemostat assay in eutrophication analyses.

RATIONAL FRAMEWORK FOR THE APPLICATION OF BIOASSAY RESULTS

The rational application of bioassay results to solve practical eutrophication problems requires information on kinetic models to describe the behavior of natural algal populations, chemical-biological interactions, physical-biological interactions, etc. The most urgently needed information is accurate kinetic descriptions for the organisms of concern, and especially for the test alga and the presumed growth rate limiting nutrients. Information on the growth kinetics of the test algal species is required to interpret the growth responses obtained by field sample assays. Knowledge of the growth kinetics of other (nuisance) algae is needed to extrapolate the bioassay findings to the natural environment.

The use of the batch culture method to determine the algal growth kinetics using various models also was found previously to be unsatisfactory [34]. On the other hand the use of chemostats to determine the kinetics of growth of *Selenastrum capricornutum* was found to be extremely satisfactory (high level of statistical significance) and the kinetic constants such as μ , K_s , and k_d were estimated accurately and the dependence of Y_P on residence time was evaluated.

OPERATIONAL CONSIDERATIONS

Equipment

The equipment needs for chemostat assays are greater than for batch assays. However, one chemostat unit is much more versatile than one batch culture flask, therefore the real difference between the two methods is probably not significant.

Simplicity

The batch assay is simpler to perform than the chemostat assay. However, this does not indicate that the needs for operating chemostat assays require the use of specialized personnel but rather that some familiarity with the assay method must be developed for satisfactory operation.

Operational Cost

Although specific cost figures have not been prepared there is probably very little difference in the operational cost for the two assay methods. However, chemostat assays require larger volumes of samples than batch assays.

Time Requirements

The average duration of a PAAP standard batch assay is from 14 to 21 days. To conduct a chemostat assay at a single residence time about 14 days is needed; however, during this period identification of the growth-limiting nutrient can be made. With batch cultures the identification of the growth-limiting nutrient needs either a separate experiment for spiking or many different flasks at the beginning; whereas with chemostats the same units are used for the assay and for identifying the growth-limiting nutrient.

THE ROLE OF BATCH AND CHEMOSTAT ASSAYS IN EUTROPHICATION ANALYSIS

Batch Assays

The linearity of cell concentration response with respect to the concentration of the growth-limiting nutrient, the reasonably adequate precision (coefficient of variation approximately 15%) of estimating biomass responses, the ease of operation, the low equipment cost

requirements, etc., indicate that batch algal assays should have some application in eutrophication analysis. However, the inferiority of the batch assay as compared to the chemostat assay suggests that the batch assay should be used primarily as a screening device and possibly as a routine assay for monitoring purposes.

With batch assays both parameters, $\hat{\mu}_b$ and $\hat{X}$, should be determined. When spiking treatments are used to determine the growth-limiting nutrient in batch assays, both parameters should be considered as it appears that $\hat{\mu}_b$ is the most sensitive parameter. Batch assays of rich natural waters may be subject to growth limitations by carbon and adequate facilities for ensuring abundant carbon supply should be used when carbon is not the nutrient of concern. By taking these precautions the batch culture should be a satisfactory method for screening investigations of natural water samples. However, it must be realized that the development of a rational framework for the application of batch assay data, even for initial screening methods is a major factor to be considered in the selection of the assay method.

Chemostat Assays

Chemostat assays have a dual role in eutrophication analyses [34]. First, they have been found to be extremely suitable both for determining the algal response of a water sample and for identifying the growth-limiting nutrient. Identification of the growth-limiting nutrient is possible by the use of enrichment techniques and/or the determination of K_s values. Second, the chemostat assay is unquestionably the method of choice for determining kinetic growth constants for the test algal species as well as for important (nuisance) algae. Thus, the chemostat assay provides not only the basic assay growth response results but also provides a conceptual basis for interpreting these in terms of the practical eutrophication problem. Kinetic data on the problem of "nuisance" organism and the rate-limiting nutrient is the ultimate objective to be reached for the rational analysis and interpretation of control measures for the practical eutrophication problem.

The chemostat assay method is recommended as the preferred method for laboratory assays and evaluation of the algal growth rate-limiting nutrient relationship. It is urgent that studies be undertaken to determine the kinetic characteristics of the test alga Selenastrum capricornutum with respect to all presumed significant nutrients (this study provided the first order estimate only for phosphorus). Similarly, kinetic descriptions for the organisms associated with algal blooms and the presumed significant nutrients also need to be developed. It is only with such data that a rational basis for eutrophication control measures can be developed.

SECTION VIII

ACKNOWLEDGMENTS

This report summarizes work accomplished by the University of California, Berkeley Research Team as a participant in the multi-laboratory Provisional Algal Assay Procedures Research Program initiated by the Joint Industry-Government Task Force on Eutrophication and sponsored by the Environmental Protection Agency.

The cooperation with other laboratories participating in this program is sincerely appreciated. The sources of results of other participating laboratories referred to in this report, are gratefully acknowledged.

This research was supported in major part by Research Grant 16010 DQB of the Environmental Protection Agency, U. S. Department of the Interior. The help provided by Dr. A. F. Bartsch and Mr. Thomas E. Maloney, the Grant Project Officer, is acknowledged with sincere thanks.

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SECTION VIII

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SECTION IX

REFERENCES

1. Hasler, A. D., "Eutrophication of Lakes by Domestic Sewage," Ecology 28, p. 383 (1947).
2. Sawyer, C. N., "Fertilization of Lakes by Agricultural and Urban Drainage," J. New England Water Works Assoc., 61, p. 109 (1947).
3. Edmundson, W. T., "Lake Eutrophication and Water Quality Management: The Lake Washington Case," Water Quality Control, Univ. of Washington Press, Seattle, Washington, p. 139 (1968).
4. Thomas, E. A., "Der Zürichsee, sein Wasser und sein Boden," Jahrb. vom Zürichsee, 17, p. 173 (1957).
5. Mackenthum, K. M., Keup, L. E., and Stewart, R. K., "Nutrients and Algae in Lake Sebasticook, Maine," J. Water Pollut. Control Fed. Research Suppl., 40, R72 (1968).
6. "Nutrient-Associated Problems in Water Quality and Treatment," Task Group 2610-P Report, J. Am. Water Works Assoc., 58, p. 1337 (1966).
7. Stewart, K. M., and Rohlich, G. A., Eutrophication - A Review, A Report to the California Water Quality Control Board, Publication No. 34, p. 188 (1967).
8. National Academy of Science, Eutrophication: Causes, Consequences, Correctives, Proceedings of a Symposium, National Academy of Sciences, Washington, D. C., p. 669 (1969).
9. Upsalla Symposium, Eutrophication in Large Lakes and Impoundments, O. E. C. D. Report No. 26, p. 560 (1970).
10. Mulligan, H. F., "Management of Aquatic Vascular Plants and Algae," in Eutrophication: Causes, Consequences, Correctives, National Academy of Sciences, Washington, D. C., p. 464 (1969).
11. Knight, A., and Ball, R. C., "Some Estimates of Primary Production Rates in Michigan Ponds," Michigan Academy of Science, Arts and Letters, 47, p. 219 (1962).
12. Kuentzel, L. E., "Bacteria, Carbon Dioxide, and Algal Blooms," J. Water Pollut. Control Fed., 41, p. 1737 (1969).

13. Legge, R. F., and Dingledein, D., "We Hung Phosphates Without A Fair Trial," Can. Res. Develop., p. 19 (March 1970).
14. "Detergent Phosphates Under Fire," Environmental Sci. Tech., 4, p. 101 (1970).
15. Bowen, D. H. M., "The Great Phosphorus Controversy," Environmental Sci. Tech., 4, p. 725 (1970).
16. Sawyer, C. N., "Discussion - Bacteria, Carbon Dioxide, and Algal Blooms," J. Water Pollut. Control Fed., 42, p. 677 (1970).
17. Abelson, P., "Excessive Emotion About Detergents," Science, 169, p. 1033 (1970).
18. Kerr, P. C., Paris, D. F., and Brochway, D. L., "The Interrelation of Carbon and Phosphorus in Regulating Heterotrophic and Autotrophic Populations in Aquatic Ecosystems," Water Pollut. Control Res. Ser. 16050 FGS 07/70, U.S. Environmental Protection Agency, (1970).
19. Shapiro, J., "A Statement on Phosphorus," J. Water Pollut. Control Fed., 42, p. 772 (1970).
20. Kuentzel, L. E., "Correction," J. Water Pollut. Control Fed., 42, p. 353 (1970).
21. Dawson, W. L., Phosphates in Detergents and the Eutrophication of America's Waters, Twenty-Third Report by the Committee on Government Operations, 91st Congress, 2nd Session, House Report No. 91-1004, U.S. Government Printing Office, Washington, D. C. (1970).
22. King, D. L., "The Role of Carbon in Eutrophication," J. Water Pollut. Control Fed., 42, p. 2035 (1971).
23. Likens, G. E., "The Limiting Nutrient Controversy," Symposium on Nutrients and Eutrophication, Am. Soc. Limnol. and Oceanog., Michigan State University, East Lansing, Michigan (1971).
24. Ryther, J. H., and Dunstan, W. M., "Nitrogen, Phosphorus, and Eutrophication in the Coastal Marine Environment," Science, 171, p. 1008 (1971).
25. Monod, J., "The Growth of Bacterial Cultures," Ann. Rev. Microbiol., 3, p. 371 (1949).
26. Caperon, J. W., The Dynamics of Nitrate Limited Growth of Isochrysis galbana Populations, Ph. D. Thesis, University of California, San Diego (1965).

27. Dugdale, R. C., "Nutrient Limitation in the Sea: Dynamics, Identification and Significance," Limnol. and Oceanog., 12, p. 685 (1967).
28. Eppley, R. W., Rogers, J. N., and McCarthy, J. J., "Half Saturation Constants for Uptake of Nitrate and Ammonium by Marine Phytoplankton," Limnol. and Oceanog., 14, p. 912 (1969).
29. Smith, F. E., "Effects of Enrichment in Mathematical Models," in Eutrophication: Causes, Consequences, Correctives, National Academy of Sciences, Washington, D. C., p. 631 (1969).
30. McGauhey, P. H., Rohlich, G. A., Pearson, E. A., Tunzi, M., Aleti, A., and Middlebrooks, E. J., First Progress Report, Eutrophication of Surface Waters - Lake Tahoe Bioassay of Nutrient Sources, Lake Tahoe Area Council, South Lake Tahoe, California, p. 178 (1968).
31. McGauhey, P. H., Rohlich, G. A., Pearson, E. A., Middlebrooks, E. J., Porcella, D. B., Aleti, A., and Tunzi, M., Eutrophication of Surface Waters - Lake Tahoe: Laboratory and Pilot Pond Studies, Lake Tahoe Area Council, South Lake Tahoe, California p. 180 (1969).
32. McGauhey, P. H., Pearson, E. A., Rohlich, G. A., Porcella, D. B., Dugan, G. L., and Middlebrooks, E. J., Eutrophication of Surface Waters - Lake Tahoe (Indian Creek Reservoir), Lake Tahoe Area Council, South Lake Tahoe, California, p. 141 (1970).
33. McGauhey, P. H., Pearson, E. A., Rohlich, G. A., Dugan, G. L., Porcella, D. B., and Middlebrooks, E. J., Eutrophication of Surface Waters - Lake Tahoe: Pilot Pond and Field Studies, Lake Tahoe Area Council, South Lake Tahoe, California, p. 109, (1970).
34. Porcella, D. B., Grau, P., Huang, C. H., Radimsky, J., Toerien, D. F., and Pearson, E. A., Provisional Algal Assay Procedures: First Annual Report, SERI Report No. 70-8, University of California, Berkeley, California, (1970).
35. Provisional Algal Assay Procedures, Joint Industry - Government Task Force on Eutrophication, P. O. Box 3011, Grand Central Station, New York, p. 62 (1969).
36. Fitzgerald, G. P., "Field and Laboratory Evaluations of Bioassays for Nitrogen and Phosphorus with Algae and Aquatic Weeds," Limnol and Oceanog., 14, p. 206 (1969).
37. Eppley, R. W., and Coatsworth, J. L., "Uptake of Nitrate and Nitrite by Ditylum bugtwellii - Kinetics and Mechanisms," J. Phycol., 4, p. 151 (1968).

38. Holm-Hansen, W., Booth, C. R., "The Measurement of Adenosine Triphosphate in the Ocean and its Ecological Significance," Limnol. and Oceanog., 11, p. 510 (1966).
39. Strickland, J. D. H., and Parsons, T. R., A Manual of Sea Water Analysis, Bulletin No. 125, Fisheries Research Board of Canada, Ottawa (1968).
40. Lorenzen, C. J., "A Method for the Continuous Measurement of *in vivo* Chlorophyll Concentration," Deep Sea Research, 13, p. 223 (1966).
41. Manny, B. A., "The Relationship Between Organic Nitrogen and The Carotenoid to Chlorophyll a Ratio in Five Freshwater Phytoplankton Species," Limnol. and Oceanog., 14, p. 69 (1969).
42. Gaarder, T., and Gran, H. H., "Investigations of the Production of Plankton in the Oslo Fjord," Rapp. et Proc. - Verb., Cons. Int. Explor. Mer., 42, p. 1 (1927).
43. Steemann-Nielsen, E., "The Use of Radio-Active Carbon (C^{14}) For Measuring Organic Production in the Sea," J. Cons. Int. Explor. Mer., 18, p. 117 (1952).
44. Goldman, C. R., "Micronutrient Limiting Factors and Their Detection in Natural Phytoplankton Populations," Memorie Ist. Ital. Idrobiol., Suppl., 18, p. 121 (1965).
45. Allen, E. J., and Nelson, E. W., "On the Artificial Culture of Marine Plankton Organisms," J. Mar. Biol. Assoc., 8, p. 421 (1910).
46. Eyster, C., "Bioassay of Water From a Concentration-Forming Marl Lake," Limnol. and Oceanog., 3, p. 455 (1958).
47. Bringmann, G., and Kühn, R., "Der Algal-Titer als Masstab der Eutrophierung von Wasser und Schlamm," Gesundheitsingenier, 79, p. 50 (1956).
48. Skulberg, O. M., "Algal Cultures as a Means to Assess the Fertilizing Influence of Pollution," In: Advances in Water Pollution Research, Water Pollution Control Federation, Washington, D. C., Vol. 1, p. 113 (1967).
49. Oswald, W. J., and Golueke, C. G., "Eutrophication Trends in the United States. A Problem?" J. Water Pollut. Control Fed., 38, p. 964 (1966).
50. Bain, Jr., R. C., "Algal Growth Assessments by Fluorescence Techniques," In: Proceedings of the Eutrophication-Biostimulation Assessment Workshop, Ed. E. J. Middlebrooks, et al., p. 37 (1969).

51. Goldman, C. R., Tunzi, M. G., and Armstrong, R., "¹⁴C Uptake as a Sensitive Measure of the Growth of Algal Cultures," In: Proceedings of the Eutrophication-Biostimulation Assessment Workshop, Ed. E. J. Middlebrooks, et al., p. 158 (1969).
52. Pearson, E. A., The Case for Continuous Flow (Chemostat) Kinetic Descriptions of Plankton-Nutrient Growth Relationships in Eutrophication Analyses, Prepared for Joint Industry Government Committee Meeting on Algae Growth Potential, Chicago, Illinois (6-8 March 1968).
53. Pearson, E. A., Middlebrooks, E. J., Tunzi, M., Aleti, A., McGauhey, P. H., and Rohlich, G. A., "Kinetic Assessment of Algal Growth," Proceedings of the Eutrophication-Biostimulation Assessment Workshop, Ed. E. J. Middlebrooks, et al., p. 56 (1969).
54. Borchardt, J. A., "Biological Extraction of Nutrients," J. Water Pollut. Control Fed., 40, p. 1739 (1968).
55. Scherfig, J., and Dixon, P. S., Results presented at PAAP Evaluation Meeting, Madison, Wisconsin, (October 1970).
56. Miller, W. E., Results presented at PAAP Evaluation Meeting, Madison, Wisconsin, (October 1970).
57. Lindemann, E., and Vollp, G. P., Eutrophication: Progress Report, Report No. PCR 762, FMC Corporation, Princeton (1970).
58. Haviland, J. R., Wilkes, F. G., Sless, J. B., Marshall, G. H., and Weiss, C. M., Provisional Algal Assay Procedures: Interim Report No. 2, Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill (1970).
59. Williams, J. M., and Yeisley, W. G., Provisional Algal Assay Procedure: Eutrophication Status Report, Lever Brothers, Edgewater, New Jersey (1970).
60. Payne, A. G., Results presented at PAAP Evaluation Meeting, Corvallis, Oregon (May 1970).
61. Sless, J. B., Haviland, J. R., Wilkes, F. G., Marshall, G. H., and Weiss, C. M., Provisional Algal Assay Procedures: Interim Report, Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill (1969).
62. Haviland, J. R., Wilkes, F. G., Marshall, G. H., and Weiss, C. M., Provisional Algal Assay Procedure: Interim Report No. 3, Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill (1970).

63. Haviland, J. R., Wilkes, F. G., Marshall, G. H., and Weiss, C. M., Provisional Algal Assay Procedure: Interim Report No. 4, Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill (1970).
64. Wilkes, F. G., Haviland, J. R., Marshall, G. H., and Weiss, C. M., Provisional Algal Assay Procedure: Interim Report No. 5, Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill (1970).
65. Haviland, J. R., Wilkes, F. G., Marshall, G. H., and Weiss, C. M., Provisional Algal Assay Procedure: Interim Report No. 6, Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill (1970).
66. Haviland, J. R., Marshall, G. H., and Weiss, C. M., Provisional Algal Assay Procedure: Interim Report No. 7, Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill (1970).
67. Haviland, J. R., Wilkes, F. G., Marshall, G. H., and Weiss, C. M., Provisional Algal Assay Procedure: Interim Report No. 8, Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill (1970).
68. Marshall, G. H., Haviland, J. R., Wilkes, F. G., and Weiss, C. M., Provisional Algal Assay Procedure: Interim Report No. 9, Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill (1970).
69. Weiss, C. M., Helms, R. W., and Marshall, G. H., The Inter-laboratory Precision Test: An Eight Laboratory Evaluation of the Provisional Algal Assay Procedure Bottle Test, Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill (1971).
70. Payne, A. G., Results presented at the PAAP Evaluation Meeting, Madison, Wisconsin, (October 1970).
71. Gerhold, R. M., Effects of Inoculum Age on Growth Rates and Final Yields in *Selenastrum capricornutum* Cultures in Full Strength and Dilute October '69 PAAP Medium, Water Resources Center, University of Wisconsin, Madison, Wisconsin (1970).
72. Gerhold, R. M., and Uttormark, P. D., Preliminary Development of Flask Assay Procedures, Water Resources Center, University of Wisconsin, Madison, Wisconsin (1970).
73. Maloney, T. E., Summary of PAAP Evaluation Meeting, October 8 - 9, 1970, National Eutrophication Research Program, Environmental Protection Agency, Corvallis, Oregon (1970).

74. Fitzgerald, G. P., Communication made at the PAAP Evaluation Meeting, Madison, Wisconsin, (October 1970).
75. Yeisley, W. G., Results presented at the PAAP Evaluation Meeting, Madison, Wisconsin, (October 1970).
76. Scherfig, J., Results presented at the PAAP Evaluation Meeting, Corvallis, Oregon, (May 1970).
77. Maloney, T. E., Algal Assay Procedure: Bottle Test, National Eutrophication Research Program, Environmental Protection Agency, Corvallis, Oregon (1971).
78. Toerien, D. F., Huang, C. H., Porcella, D. B., Radimsky, J., and Pearson, E. A., The Role of Chemostat Assays in Eutrophication Analysis, Presented at the 21st AIBS Meeting, Bloomington, Indiana (1970).
79. Fencel, Z., "A Uniform System of Basic Symbols for Continuous Cultivation of Microorganisms," Folia Microbiol., 8, p. 192 (1963).
80. Fencel, Z., "Theoretical Analysis of Continuous Culture Systems," In: Theoretical and Methodological Basis for Continuous Culture of Microorganisms, Ed. I. Malek and Z. Fencel, Academic Press, New York (1966).
81. Michaelis, L., and Menten, M. L., "Die Kinetik der Invertinwirkung," Biochem. Z., 49, p. 333 (1913).
82. Monod, J., Reserches sur la Croissance des Cultures Bacteriennes, Paris, Hermann et C^{ie} (1942).
83. Stewart, M. J., Reaction Kinetics and Operational Parameters of Continuous Flow Anaerobic Fermentation Processes, SERL Publication No. 4, IER Series 90, University of California, Berkeley (1958).
84. Agardy, F. J., Cole, R. D., and Pearson, E. A., Kinetics and Activity Parameters of Anaerobic Fermentation Systems, SERL Report No. 63-2, University of California, Berkeley (1963).
85. Andrews, J. F., Cole, R. D., and Pearson, E. A., Kinetics and Characteristics of Multistage Methane Fermentations, SERL Report No. 64-11, University of California, Berkeley (1964).
86. Teissier, G., "Les Lois Quantitatives de la Croissance," Ana. Physiol. Physiocochim. Biol., 12, p. 527 (1936).
87. Schulze, K. L., and Lipe, R. S., "Relationship Between Substrate Concentration, Growth Rate, and Respiration Rate of Escherichia coli in Continuous Culture," Archiv Mikrobiol., 48, p. 1 (1964).

88. Schulze, K. L., "The Activated Sludge Process as a Continuous Flow Culture," Water Sew. Works, 111, p. 526 (1964).
89. Schulze, K. L., "The Activated Sludge Process as a Continuous Flow Culture," Water Sew. Works, 112, p. 11 (1965).
90. Shelef, G., Oswald, W. J., and Golueke, C. G., Kinetics of Algal Systems in Waste Treatment - Light Intensity and Nitrogen Concentration as Growth Limiting Factors, SERL Report No. 68-4, University of California, Berkeley (1968).
91. Moser, H., The Dynamics of Bacterial Populations Maintained in the Chemostat, Publication No. 614, Carnegie Institute of Washington (1958).
92. Contois, D. E., "Kinetics of Bacterial Growth - Relationship Between Population Density and Specific Growth Rate of Continuous Cultures," J. Gen. Microbiol., 21, p. 40 (1959).
93. Gerloff, G. C., and Skoog, F., "Nitrogen as a Limiting Factor for the Growth of Microcystis aeruginosa in Southern Wisconsin Lakes," Ecology, 38, p. 556 (1957).
94. Birge, E. A., and Juday, C., "The Inland Lakes of Wisconsin - The Plankton. I. Its Quantity and Chemical Composition," Bull. Wis. Geol. Nat. Hist. Surv., 64, p. 219 (1922).
95. Mackenthum, K. M., and Ingram, W. M., Biological Associated Problems in Fresh Water Environments, USDI, FWPCA (1967).
96. Fuhs, G. W., "Phosphorus Content and Rate of Growth in the Diatoms Cyclotella nana and Thalassiosira fluvialilis," J. Phycol. 5, p. 312 (1969).
97. Rodhe, W., "Environmental Requirements of Fresh-Water Plankton Algae," Symb. bot. Upsal., 10, p. 1 (1948).
98. Mackereth, F. J. H., "Phosphorus Utilization by Asterionella formosa Hass," J. Exp. Bot., 4, p. 296 (1953).
99. Marr, A. G., Nilson, E. H., and Clark, D. J., "The Maintenance Requirements of Escherichia coli," Ann. N. Y. Acad. Sci., 102, p. 536 (1963).
100. Hetling, L. J., Washington, D. R., and Rao, S. S., Kinetics of the Steady State Bacterial Culture. II. Variations in the Rate of Synthesis, Rennselaer Polytech. Inst. Troy, New York (1964).
101. Mor, J. R., and Fiechter, A., "Continuous Cultivation of Saccharomyces cerevisiae. I. Growth on Ethanol under Steady-State Conditions," Biotech. Bioeng., 10, p. 159 (1968).

102. Mor, J. R., and Fiechter, A., "Continuous Cultivation of Saccharomyces cerevisiae. II. Growth on Ethanol under Transient-State Conditions," Biotech. Bioeng., 10, p. 787 (1968).
103. Zabat, M., Kinetics of Phosphorus Removal by Algae, Ph. D. Thesis, University of California, Berkeley (1971).
104. Goldberg, E. D., Walker, T. J., and Whisenand, E., "Phosphate Utilization by Diatoms," Biol. Bull. 101, p. 274 (1951).
105. Strickland, J. D. H., and Parsons, T. R., A Manual of Sea Water Analysis, Bulletin No. 125, Fisheries Research Board of Canada, Ottawa (1968).
106. Standard Methods for the Examination of Water and Waste Water, 12th Ed., American Public Health Association, New York (1965).
107. Solorzano, L., "Determination of Ammonia in Natural Waters by the Phenolhypochlorite Method," Limnol. Oceanog., 14, p. 799 (1969).
108. Maloney, T. E., Summary of PAAP Evaluation Meeting at Corvallis, May 1970, National Eutrophication Program, Corvallis, Oregon (1970).
109. Dixon, P. S., Personal Communication (1970).
110. Water Quality Criteria, Report of the National Advisory Committee to the Secretary of the Interior, Federal Water Pollution Control Administration, Washington, D. C. (1968).
111. Holm-Hansen, O., "Environmental and Nutritional Requirements for Algae," In: Proceedings of the Eutrophication-Biostimulation Assessment Workshop, Ed., E. J. Middlebrooks, et al., p. 98 (1969).

SECTION X

PUBLICATIONS AND PATENTS

Publications

1. Porcella, D. B., Grau, P., Huang, C. H., Radimsky, J., Toerien, D. F., and Pearson, E. A., "Provisional Algal Assay Procedures - First Annual Report," SERL Report No. 70-8, Sanitary Engineering Research Laboratory, University of California, Berkeley (1970).
2. Toerien, D. F., Huang, C. H., Porcella, D. B., Radimsky, J., and Pearson, E. A., "The Role of Chemostat Assays in Eutrophication Analysis," Presented at the 21st AIBS Meeting, Bloomington, Indiana (1970).

Patents

None

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NOT REPRODUCIBLE

SECTION XI

GLOSSARY

A	Nutrient content of individual cell at cell age θ in chemostat
A_o	Nutrient content of individual cell at infinite cell age in chemostat
B	Growth parameter in Contois' function
C_v	Coefficient of variation
F	Flow rate, volume/time
f_e	Fraction of excess nutrient (phosphorus) uptake
g	Generation time, time
k	A growth constant in Teissier's Exponential Model
k_l	Nutrient utilization rate, time^{-1}
k_d	Specific organism decay rate, time^{-1}
K_A	Nutrient utilization constant in exponential function, time
K_B	Nutrient utilization constant in hyperbolic function, time
K_a	A growth constant in modified Teissier's Model, mass/volume
K_c	A rate constant in Blackman First Order Function
K_s	Half saturation constant, mass/volume
n	Number of events
N	Number of generations
p	Probability of occurrence of the event
P	Population density
P_r	Rate of predation of algal cells
q	Nutrient removal velocity, time^{-1}
S	Essential nutrient concentration, mass/volume
S_l	Nutrient concentration in chemostat or in the effluent, mass/volume
S_o	Initial nutrient concentration, mass/volume
S_B	Suspension rate of benthic algae
S_c	Nutrient saturation concentration, mass/volume
S_e	Excess nutrient uptake concentration, mass/volume
S_g	Nutrient concentration utilized by cells for their growth, mass/volume

S_s	Net settling rate of algal cells
S_r	Resuspension rate of algal cells
t	Time
t_e	Time length of batch growth to reach zero growth rate
V	Chemostat volume, volume
X	Organism concentration, mass/volume
X_0	Initial concentration of organisms, mass/volume
X_1	Organism concentration in chemostat or effluent, mass/volume
X_2	Concentration of higher organisms responsible for algal predation
X_3	Algal cell concentration available for predation by higher organisms
X_4	Concentration of planktonic algae on the bottom of water body
X_5	Concentration of benthic algae
$\hat{X}$	Maximum concentration of organisms, batch, mass/volume
$X_{\max}$	Maximum steady state cell concentration in chemostat, mass/volume
Y	Growth yield coefficient
Y_n	Net cell yield coefficient
$Y_{\max}$	Maximum cell yield coefficient
θ	Hydraulic residence time, time
θ_c	Mean cell age, time
θ_w	Residence time at which "washout" occurs, time
μ	Specific growth rate, time ⁻¹
μ_b	Specific growth rate, batch, time ⁻¹
$\hat{\mu}$	Maximum specific growth rate, time ⁻¹
$\hat{\mu}_b$	Maximum specific growth rate, batch, time ⁻¹

SECTION XII

APPENDICES

	<u>Page No.</u>
A. Air Supply System for Batch and Chemostat Cultures	184
B. Computer Program G2 CAL NLIN	186
C. Batch Culture Assay Results	196
Table C-I: First Interlaboratory Precision Test	196
Table C-II: Second Interlaboratory Precision Test	197
Table C-III: Clear Lake Study	198
Table C-IV: Secondary Effluent Enrichment of Clear Lake Water	199
Table C-V: Clear Lake Enrichment (Spiking) Tests	200
Table C-VI Sacramento River Study	202
Table C-VII Secondary Effluent Enrichment of Sacramento River Water	203
Table C-VIII Sacramento River Enrichment (Spiking) Tests	204
D. Chemostat Operation Data	206

APPENDIX A

AIR SUPPLY SYSTEM FOR BATCH AND CHEMOSTAT CULTURES

1. Flow rates required.

- a) $0.12 \text{ ft}^3 \text{ hr}^{-1}$ in each batch culture
- b) $0.50 \text{ ft}^3 \text{ hr}^{-1}$ in each chemostat culture.

It is expected that it may be necessary to add carbon dioxide to the air stream and/or to vary the flow rates to maintain the pH in the desired range of 7.0 to 8.3.

2. Mean residence times in glass wool filters.

The mean residence times can be calculated as illustrated:
Assume minimum reasonable decontamination occurs in 0.25 minute.
Then for a 2-inch diameter PVC pipe:

$$\text{Area, } A = 19.6 \text{ cm}^2 \approx 20 \text{ cm}^2$$

$$\text{Total Air Flow Rate, } Q = (\# \text{ batch})(0.12) + (\# \text{ chemostats})$$

$$(0.5 \text{ ft}^3 \text{ hr}^{-1})$$

$$= (25)(0.12) + 16(0.5)$$

$$= 11 \text{ ft}^3 \text{ hr}^{-1}$$

$$= 312 \text{ L hr}^{-1}$$

$$= 312 \times 10^3 \text{ cm}^3 \text{ hr}^{-1}$$

$$\text{Air Velocity, } v = \frac{Q}{A} = \frac{312 \times 10^3 \text{ cm}^3 \text{ hr}^{-1}}{20 \text{ cm}^2}$$

$$= 16 \times 10^3 \text{ cm hr}^{-1}$$

$$= 270 \text{ cm min}^{-1}$$

For plug flow the residence time in a 2-inch diameter PVC pipe,
 $t = L/v$

$$0.25 = \frac{L}{270}$$

$$L = (0.25)(270) \text{ cm}$$

= 68 cm long tube

to provide for variation a 90 cm tube can be used.

3. Filtration, trapping and humidification.

- a) Glass wool - particulate filtration (replace every 6 months).
- b) Activated carbon - organic trap (replace every 6 months).
- c) 0.1 N H_2SO_4 in distilled water - trap NH_3 (replace after 20 percent loss of acidity).
- d) Glass bead trap directly after acid wash - prevent carryover of acid.
- e) Distilled water trap - humidify (replace after 20 percent loss of volume).
- f) Glass bead trap after water trap - prevent carryover of water droplets.
- g) Mixing flask for air and carbon dioxide after water trap and glass bead trap.

4. Tubing size and flow measurement.

- a) The manifold size specified will provide adequate flows for tubing sizes <0.5 mm ID. Tygon tubing should be ≥ 6 mm (1/4 in.) ID.
- b) At least one flowmeter for the batch manifold and one for the chemostat manifold must be provided. The individual chemostats should each have a flowmeter.

APPENDIX B
COMPUTER PROGRAM G2 CAL NLIN

IDENTIFICATION

Code Designation:	G2 CAL NLIN
Title:	Nonlinear Regression
Author:	R. M. Baer, Computer Center University of California, Berkeley
Date:	June 1967
Specification:	CDC 6400 SCOPE 2.0 Operating System
Decks Included:	TESTL (main program) INDATA SETUP GAUSS DMPMAT DERIV BTEST FINALE LINEOF
Additional Decks Needed:	YCOMP (written and supplied by the user)

PURPOSE

To find the values of the parameters $B_1, B_2, \dots$, which minimize the sum of squares

$$S = \sum [z_1 - G(z_2, z_3, \dots; B_1, B_2, \dots)]^2 \quad *$$

where the summation is over a collection of "data points," each point consisting of a set of "observed" values for the variables $(z_1, \dots, z_m)$,

*Or the relative sum of squares (either with respect to the observed values Z_1 or the calculated values $G(\dots)$).

$m \geq 2$, where the fitting parameters $B_1, B_2, \dots$ and the observables $z_1, z_2, \dots$ satisfy an equation

$$z_1 = G(z_2, \dots; B_1, B_2, \dots).$$

Alternately the program may be used to find a "best" solution to a set of nonlinear equations for which the number of equations is greater than or equal to the number of unknowns.

METHOD

The procedure which is used here replaces the function G by its first order Taylor expansion (in the B_j), solves for the minimum of S (which is being approximated by a quadratic) by solving the set of linear equations which express the fact that the approximation of S should have zero gradient. The values of the parameters B_j are changed accordingly; and then the whole procedure is iterated until the corrections for the B_j become negligible or the number of iterations exceeds a limit.

References

1. Collatz, L., Numerische und graphische Methoden. Handbuch d. Physik, Band II, Berlin (1955).
2. Hartley, H. O., The Modified Gauss-Newton Method for the Fitting of Non-linear Regression Functions by Least Squares. *Technometrics* 3, 269-280 (1961).
3. Baer, R. M., Nonlinear Regression and the Solution of Simultaneous Equations. *Comm. ACM* 5, No. 7 (1962).

USAGE

I. The Complete NLIN Program

The embedding routine (main routine) of this program has as its purpose 1) introducing and checking options and data, and 2) calling the two major subroutines, GAUSS (along with SETUP) and FINALE.

GAUSS is the subroutine which seeks the minimizing values of the parameters, and FINALE is the subroutine which produces a tabulation of the results of fitting for current values of the parameters B_j .

Either of these subroutines may be used independently for specific needs: in solving certain automatic control problems, programs may use only GAUSS and SETUP since a tabulation of the goodness of fit at each fitting would result in too much output and would slow down the control program; on the other hand, some users want to see merely a tabulation of the fitting of data by an equation which is nonlinear in the parameters and so use only FINALE — the parameters in their case have fixed values (e.g., are certain natural constants) and what is of interest is how good the data are. The usage of these subroutines is covered in later sections. The following two sections cover the usage of the complete library program.

A. Data Input to the Embedding Program (Library Version)

Each problem to be processed by NLIN requires a data deck, described below. Several problem decks may be stacked. Processing will be terminated by a control card carrying a zero. The data deck consists of

- i) a control card — See B.1
- (on option) ii) card(s) with starting guesses for the parameters $B_1, B_2, \dots$ — See B.2
- (on option) iii) cards with the values of the "data point" observables $Z_1(N), Z_2(N), \dots, n=1, 2, \dots, NPTS$. (The data point cards may be omitted. See B.1, B.3)

B. Data Entries

1. Each problem requires its own control card, from which the program reads ten control values. These ten (integer type) quantities are read according to FORMAT (10I6). The program identifiers for these quantities are (CNTROL(I), I=1, 10). The values must be set in accordance with the following:

CNTROL (1) is the number of parameters B_i with respect to which the sum of squares is to be minimized. If this variable is given a positive value then the program expects to read a data card carrying guesses at the values of the B_i . If a minus sign is prefixed to the value of CNTROL(1) then the program skips the command which reads in values for the B_i (and so there should be no cards carrying values for the B_i).

CNTROL (2) is the number of data points, Z , which are to be used in the minimization. If CNTROL(2) is

positive, the program expects to read data cards carrying the values of the Z_i . If CNTROL(2) has a minus sign prefixed to its value, then the program skips the commands which read values for the data points. For example, a data point might consist of (Z_1, Z_2, Z_3) ; and there might be 4 data points in the particular problem. Then the value of CNTROL(2) would be 4, unless the same data points are to be used as may have already been read-in from a preceding problem — in which case CNTROL(2) may be given the value -4 and duplicating the data cards carrying values for the data points may be skipped.

- CNTROL (3) is the number of Z_i per data point; in the example each data point is a triplet (Z_1, Z_2, Z_3) so that CNTROL(3) is 3.
- CNTROL (4) is an upper bound on the number of iterations. (A conservative value is $5 + 2k$ where k is the number of B_i).
- CNTROL (5) determines the printout of intermediate iterations as follows: Let $N = J + 2K + 4L$, where J , K , and L can be either 0 or 1. J refers to the printout of input data, K to the printing of consecutive iterations (sum of squares and parameters B), and L to the current matrices for the normal equations. A value of 0 indicates that no printing is desired; a value of 1 indicates that the intermediate printing is to be done. Set $\text{CNTROL}(5) = N$.
- CNTROL (6) determines whether the sum of squares, or a relative sum of squares is to be minimized. The value 0 results in minimization of the ordinary sum of squares; the value 1 results in the minimization of the relative sum of squares with respect to the observations; -1, results in the minimization of the relative sum of squares with respect to the calculated function.
- CNTROL (7) is set by the program and equals the current iteration number.
- CNTROL (8) is set by the program and indicates the nature of termination.

*CNTROL (9) is at the user's disposal and may be used to indicate the computation (i.e., the fitting function) which is to be used.

CNTROL (10) is at the user's disposal. A value of 0 permits free correction of the parameters at each iteration. A value of 1 permits the parameters to be corrected by at most ten percent per iteration.

2. Parameter Entries (in format 8F10.4):

$$B_1 \quad B_2 \quad \dots \quad B_{NB}$$

On the control card the variable NB represents the number of parameters to be fitted. The card(s) carrying starting guesses for these parameters immediately follow the control card.

3. Data Point Entries for each data point, n, are (in format 8F10.4):

$$Z_1(N) \quad Z_2(N) \quad \dots \quad Z_m(N)$$

where $Z_1(N)$ is the entry for the dependent variable and where m is the number of entries for each data point (with a maximum $m = 8$ corresponding to the Dimension $Z(8,200)$).

The routines will compute on the basis of a matrix of all the data, viz.,

$$Z_1(1), Z_2(1), \dots, Z_m(1)$$

for the first data point, and

$$Z_1(2), Z_2(2), \dots, Z_m(2)$$

for the second data point, etc., until finally

$$Z_1(NPTS), Z_2(NPTS), \dots, Z_m(NPTS)$$

for the last data point.

On the control card the variable NPTS controls the number of data points to be read and/or fitted. A maximum of 200 points is permissible with the library version of NLIN.

*A negative value of CNTROL(9) results in an output format suitable for teletype when Common File version of NLIN is used.

II. Function YCOMP

The user must supply a function routine representing the nonlinear equation(s)

$$z_1 = G(z_2, \dots; B_1, B_2, \dots)$$

for his particular problem.

The function must be a (FORTRAN IV) function subprogram named YCOMP with the following basic form:

```
FUNCTION YCOMP (N,B,Z)
  DIMENSION B(24), Z(8,200)
  YCOMP = ..... The expression for G(z2,...; B1,B2,...)
  RETURN
END
```

YCOMP is computed for the n-th data point: thus the fixed point argument N in the calling sequence. All references to the $z_i(N)$ must be of the form: $Z(2,N)$, $Z(3,N)$, etc. All references to the B_i must be of the form: $B(1)$, $B(2)$, etc. The array dimensions for $z_i(N)$ must agree with those of the embedding program. Those given are for the library version.

III. Example

Suppose that four experiments have been performed in which flux, denoted F, is measured as a function of two field strengths, denoted E and H. A tabulation of the data gives

F	E	H
1.6667	1.0000	2.0000
1.5000	2.0000	3.0000
1.4000	3.0000	4.0000
1.3333	4.0000	5.0000

It is known that the relation among the variables is given by

$$F = \frac{B_1 + B_3 H}{B_2 + E}$$

There is some uncertainty as to the values of the parameters B_1 , B_2 , and B_3 in this expression but it is believed that all three have values not too far from 1.5. If GAUSS, together with its accompanying subprograms and embedding program, were to be used to find least-squares values for

B_j , the usual deck would probably consist of a small FORTRAN source deck (for YCOMP), followed by the binary decks for the embedding program and GAUSS and FINALE (and DERIV), the binary deck for F4 CAL LINEQS, and finally (control and) data cards. At present the binary deck for NLIN contains LINEQF, so that the user need only add his YCOMP, control, and data cards.

Thus, there are three parameters B, four data points, three variables (F, E, and H). Suppose we want a limit of 40 iterations, print-out of the input data and sum of squares and parameters and current matrices of normal equations, at each iteration (so that CNTROL(5) = 7), and minimization of sum of squares of relative errors.

YCOMP and the control and data cards would appear as follows:

```

FUNCTION YCOMP(N,B,Z)
DIMENSION B(24), Z(8,200)
YCOMP = (B(1) + B(3) * Z(3,N))/(B(2) + Z(2,N))
RETURN
END
7-8-9 card to introduce data

  3   4   3.40   7           CONTROL
1.5   1.5   1.5 B(J) starting guesses
1.6667 1.0   2.0 Z(I,1) observations for point 1
1.5    2.0   3.0 Z(I,2) observations for point 2
1.4    3.0   4.0 Z(I,3) observations for point 3
1.3333 4.0   5.0 Z(I,4) observations for point 4
0                                CONTROL STOP

```

A comparison between NLIN's operation when supplied exact data and when given less exact data is available in the writeup for G2 BC NLIN. There is also a comparison of performance when a large number of parameters to be fitted is used instead of a small number.

IV. Sources of Error

A. Overflows

Overflow from products and sums may be caused by exceedingly bad guess for the $B(J)$.

The use of very large parameters or data may cause overflows also. Scaling the input data so that the $Z_i(N)$ are close to unit may help.

- B. Singularity of normal equations is usually due to a miscoded function (such as the inclusion of Some B_j value which is not a parameter).

- C. The final values of $B(J)$ are the best found by GAUSS but not necessarily the solution to the problem.

V. Subroutine GAUSS

The duty of subroutine GAUSS is to find values for the parameters B_j which minimize the sum of squares of differences between the fitting functions and the dependent observable. GAUSS may find the minimizing values and return, or may find better values than the starting guesses but be forced, by the limit on the number of iterations, to return before the minimizing values have been discovered, or may be stymied by the occurrence of a set of normal equations which cannot be solved. In any case, upon return of control from GAUSS to the calling routine the set of values returned for the parameters B_j are always those giving whatever minimum sum of squares has been discovered.

GAUSS is invoked by the call

CALL GAUSS (CNTROL, B,Z, MDIM, NDIM, BDIM, A)

where

MDIM, NDIM, BDIM, and CNTROL are fixed-point variables and B, Z, and A are floating point variables.

Subroutine SETUP is invoked by the call

CALL SETUP (CNTROL,B,Z,SUMSQ,MDIM,NDIM,BDIM)

with the variables essentially the same as above. Subroutines GAUSS and SETUP set up the normal equations and solve them (using F4 CAL LINEQS), correct the current values of the parameters B_j , check to see whether the new values of the B_j result in a smaller sum of squares, and, if so, iterate the procedure. In the event that the new values of the parameters fail to give a smaller sum of squares, the parameters are reset to values which give the minimum thus-far-discovered sum of squares, are corrected by scaled-down amounts of the original corrections, and the sum of squares is again tested.

These iterations proceed until one of four possible terminating conditions occurs. GAUSS will then return control to the calling program with the flag. CNTROL(8), set to indicate the terminating condition:

	Condition
CNTROL(8) = 0	If convergence (a change of less than 10^{-5}) of the values of the parameters B_j occurs.
CNTROL(8) = 1	If the B_j iterations proceed until the scaled down corrections to the parameters become negligible without producing a new minimum in the sum of squares.

CNTROL(8) = 2 If subroutine GAUSS finds that the normal equations cannot be solved. The offending matrices are recorded.

CNTROL(8) = 3 If the number of iterations exceeds LIMIT or CNTROL(4).

VI. Subroutine FINALE

FINALE is invoked by the call

```
CALL FINALE(CNTROL,B,Z,MDIM,NDIM)
```

where all arguments are the same as those used for the GAUSS call. The subroutine, when supplied with the indicated arguments, gives a tabulation of results. FINALE first gives a tabulation of the values of the dependent variable (i.e., z_1 or Y OBSERVED as it is referred to, in the output) for the totality of data points. Corresponding to each of these values is the corresponding value of the fitting function, evaluated for the supplied B and Z arrays, the difference between these two values, and the percent deviation of the observed value from the calculated value of the dependent variable.

Then there follows a listing of the average and maximum deviation. It may be noted that, following the values for the maximum deviation and the maximum percent deviation, there are two integer entries. These are the indices of the data points which gave rise to these maximum deviations. Often such points represent errors in the data and it may be worthwhile to repeat the analysis with the offending points corrected or deleted.

VII. Function DERIV

This subprogram, FUNCTION DERIV(K,N,B,Z,BDIM), supplies the partial derivative of YCOMP with respect to the variable B(K), evaluated at the n-th data point. The version supplied with the program computes the value required by a symmetric differencing scheme. However, the user may always compile his own version to supply the exact partial derivatives. In small test problems, the final values obtained for the parameters are accurate to at least six significant figures; so the general purpose version of DERIV will be adequate for most problems.

VIII. Other Subroutines

A. Subroutine INDATA, invoked by the call

```
CALL INDATA(CNTROL, B,Z,MDIM,NDIM,BDIM)
```

reads in the data and prints titles.

- B. Subroutine BTEST, invoked by the call

CALL BTEST (CNTROL,B,BDIM)

Tests the amount of change in the parameters B.

- C. Subroutine DMPMAT, invoked by the call

CALL DMPMAT(CNTROL,BDIM,A,B(1,3))

prints intermediate iterations according to the user's specifications in CNTROL(5).

Subroutines Needed:

User's own YCOMP

TIMING

The sample problem used 3.226 seconds of central processor time and 8.712 seconds of peripheral processor time. See the writeup for G2 BC NLIN for comparisons of the time and accuracy yielded in using 6 B parameters and in using 4 B parameters (a simplified equation).

SIZE

Embedding routine	3316 ₈ locations
INDATA	601 ₈ locations
SETUP	372 ₈ locations
GAUSS	464 ₈ locations
FINALE	454 ₈ locations
DMPMAT	141 ₈ locations
DERIV	120 ₈ locations
LINEQF	306 ₈ locations

196

APPENDIX C

TABLE C-11. SECOND INTERLABORATORY PRECISION TEST

100% PAAP																			
Flask A1					Flask A2					Flask A3					Flask A4				
Day	pH	CC	SS	TCV	pH	CC	SS	TCV		pH	CC	SS	TCV		pH	CC	SS	TCV	
0	7.0	0.001	-	-	7.0	0.001	-	-		7.0	0.001	-	-		7.0	0.001	-	-	
3	7.0	0.021	F	-	6.9	0.007	F	-		6.9	0.006	F	-		7.0	0.003	F	-	
5	7.8	0.459	40.88	-	8.0	0.547	52.8	-		7.9	0.398	35.66	-		8.0	0.045	4.28	-	
7	8.2	3.272	242.11	-	8.0	2.124	159.3	-		8.2	2.82	229.16	-		8.0	0.071	6.53	-	
9	8.6	9.909	678.8	-	8.7	12.627	1111.17	-		9.0	9.64	72.96	-		8.0	0.124	11.33	-	
13	8.1	48.700	3068.08	-	8.2	19.353	1219.26	-		8.1	20.25	1306.0	-		8.1	0.321	26.15	-	
17	8.3	27.773	1642.7	-	8.5	31.227	1904.8	-		8.5	35.64	2174.1	-		8.4	0.193	17.05	-	
21		32.508	381.3	2048.0		29.058	377.5	1874.0			27.37	400.0	1582.0		0.188	6.0	18.986	-	
																		35.226	350.0
																			2131.2

30% PAAP																			
Flask B1					Flask B2					Flask B3					Flask B4				
Day	pH	CC	SS	TCV	pH	CC	SS	TCV		pH	CC	SS	TCV		pH	CC	SS	TCV	
0	7.0	0.001	-	-	7.0	0.001	-	-		7.0	0.001	-	-		7.0	0.001	-	-	
3	7.0	0.006	F	-	6.9	0.007	F	-		6.9	0.010	F	-		6.6	0.015	F	-	
5	8.1	0.176	18.69	-	8.0	0.679	62.46	-		7.8	1.22	129.94	-		7.3	0.572	54.96	-	
7	7.7	0.666	59.9	-	7.7	4.938	335.8	-		7.9	4.99	334.60	-		7.7	4.132	276.87	-	
9	8.5	3.357	288.68	-	8.5	5.524	383.9	-		8.2	6.87	470.96	-		8.3	5.574	557.43	-	
13	8.0	8.463	842.05	-	8.0	7.195	496.48	-		7.7	6.67	437.37	-		7.7	6.952	472.7	-	
17	8.4	6.370	423.62	-	8.2	6.606	495.9	-		8.2	8.52	549.44	-		8.1	9.535	524.55	-	
21		7.485	158.0	523.9		7.137	178.0	478.2			8.96	153.5	586.8		9.859	162.5	501.39	-	
																		8.58	162.0
																			609.24

10% PAAP																			
Flask C1					Flask C2					Flask C3					Flask C4				
Day	pH	CC	SS	TCV	pH	CC	SS	TCV		pH	CC	SS	TCV		pH	CC	SS	TCV	
0	7.0	0.001	-	-	7.0	0.001	-	-		7.0	0.001	-	-		7.0	0.001	-	-	
3	6.7	0.002	F	-	6.7	0.004	F	-		6.8	0.004	F	-		6.1	0.002	F	-	
5	7.2	0.018	2.27	-	7.8	0.016	2.52	-		7.7	0.004	0.398	-		7.7	0.007	0.858	-	
7	7.7	0.022	1.98	-	7.5	0.057	5.66	-		7.3	0.007	0.745	-		7.2	0.008	0.888	-	
9	7.6	0.026	2.71	-	7.7	0.233	24.17	-		7.3	0.011	0.99	-		7.4	0.016	1.88	-	
13	8.0	0.036	3.136	-	8.0	2.69	211.18	-		7.3	0.021	1.585	-		7.1	0.019	2.06	-	
17	7.6	0.039	2.953	-	7.5	1.783	141.7	-		7.3	0.016	1.151	-		7.2	0.021	1.121	-	
21		0.012	1.83	1.209		1.729	47.7	159.1			0.008	1.55	0.764		0.021	15.8	2.345	-	
																		2.524	62.9
																			163.4

1% PAAP																			
Flask D1					Flask D2					Flask D3					Flask D4				
Day	pH	CC	SS	TCV	pH	CC	SS	TCV		pH	CC	SS	TCV		pH	CC	SS	TCV	
0	7.0	0.001	-	-	7.0	0.001	-	-		7.0	0.001	-	-		7.0	0.001	-	-	
3	5.8	0.0002	F	-	5.6	0.0005	F	-		5.6	0.0001	F	-		5.6	0.0003	F	-	
5	7.1	0.0024	0.304	-	7.5	0.0019	0.268	-		7.2	0.0007	0.088	-		7.0	0.0016	0.137	-	
7	7.2	0.0124	1.377	-	7.0	0.0034	0.395	-		7.1	A	F	-		6.9	A	F	-	
9	7.1	A	1.275	-	7.1	0.0016	0.198	-		7.1	0.0022	0.286	-		6.9	0.0024	0.269	-	
13	7.2	0.0042	0.363	-	7.1	0.0103	1.074	-		7.0	0.0033	0.197	-		6.9	0.0045	0.286	-	
17	7.4	0.0046	1.363	-	7.3	0.0018	0.379	-		7.2	0.0064	0.334	-		7.0	0.0049	0.395	-	
21		0.0040	B	0.633		0.0018	B	0.216			0.0064	B	0.838		0.0049	B	0.578	-	
																		0.0029	B
																			0.316

R = spoiled

B = too low to measure

CC = cell count $\times 10^3/\text{ml}$ TCV = cell volume concentration $\times 10^6/\text{ml}$

F = instrument in repair

APPENDIX C

TABLE C-III. CLEAR LAKE STUDY

100% Clear Lake					100% Clear Lake					100% Clear Lake				
Day	pH	CC ^b	TCV ^c	SS ^d	pH	CC	TCV	SS		pH	CC	TCV	SS	
0		0.052	4.6			0.057	5.5				0.093	10.3		
2	8.0	0.114	14.3		7.9	0.102	12.5			8.0	0.107	13.7		
3	8.4	0.118	15.2		8.4	0.133	16.8			8.4	0.113	15.9		
4	8.1	0.394	38.0		8.1	0.407	38.7			8.1	0.429	41.0		
5	7.1	0.479	42.7		7.9	0.490	44.1			7.8	0.506	45.5		
6	7.9	0.467	40.4		8.1	0.497	42.5			8.3	0.512	44.3		
7	8.1	0.704	72.5		8.2	0.506	46.5			8.4	0.565	53.1		
10	6.9	0.541	47.6	41.3	7.9	0.562	48.3	36.4		8.2	0.616	55.7	39.6	

66.7% Clear Lake ^a					66.7% Clear Lake					66.7% Clear Lake				
Day	pH	CC	TCV	SS	pH	CC	TCV	SS		pH	CC	TCV	SS	
0		0.078	8.5			0.073	7.9				0.094	10.1		
2	7.8	0.054	6.9		7.8	0.078	10.0			7.8	0.082	10.9		
3	8.3	0.088	11.3		8.2	0.125	15.6			8.2	0.093	12.1		
4	8.1	0.295	26.1		8.1	0.311	26.6			8.1	0.303	27.2		
5	7.9	0.348	29.1		8.0	0.416	38.4			8.0	0.406	36.3		
6	8.3	0.381	30.7		8.2	0.375	29.4			8.3	0.393	38.0		
7	8.2	0.472	40.6		8.2	0.426	35.2			8.1	0.494	50.6		
10	8.1	0.443	36.8	30.0	7.9	0.477	39.3	28.0		7.9	0.436	35.6	24.1	

33.3% Clear Lake ^a					33.3% Clear Lake					33.3% Clear Lake				
Day	pH	CC	TCV	SS	pH	CC	TCV	SS		pH	CC	TCV	SS	
0		0.022	2.2			0.019	1.7				0.020	1.8		
2	7.9	0.033	4.8		7.9	0.027	4.1			8.0	0.027	4.0		
3	8.2	0.080	8.6		8.1	0.070	7.9			8.0	0.066	7.4		
4	8.1	0.131	13.5		8.1	0.123	12.0			8.1	0.122	12.6		
5	7.8	0.189	17.4		7.6	0.168	14.8			7.5	0.190	18.3		
6	8.2	0.335	20.0		8.3	0.232	20.3			8.3	0.216	17.8		
7	7.9	0.285	28.9		7.8	0.232	19.1			7.7	0.252	20.9		
10	6.5	0.356	28.4	28.2	6.9	0.306	24.7	25.0		7.6	0.310	25.0		

30% PAAP Control					30% PAAP Control					30% PAAP Control				
Day	pH	CC	TCV	SS	pH	CC	TCV	SS		pH	CC	TCV	SS	
0		0.010	0.6			0.009	0.6				0.010	0.7		
2	7.5	0.018	2.6		7.5	0.019	2.5			7.6	0.015	1.6		
3	8.2	0.053	5.1		8.1	0.054	5.0			8.0	0.025	2.9		
4	8.6	1.028	76.6		7.5	1.121	80.7			7.6	0.822	56.3		
5	7.8	2.328	178.1		7.5	2.749	233.6			7.9	1.566	143.3		
6	8.2	4.318	405.9		8.3	4.567	408.8			7.6	3.214	287.7		
7	8.1	6.155	538.6		7.9	6.192	504.6			7.9	6.076	607.6		
10	7.5	7.216	570.1	152.6	7.4	7.006	514.9	151.8		7.7	7.081	501.0	140.5	

Clear Lake + 9% PAAP					Clear Lake + 9% PAAP					Clear Lake + 9% PAAP				
Day	pH	CC	TCV	SS	pH	CC	TCV	SS		pH	CC	TCV	SS	
0		0.123	12.8								0.110	12.3		
2	7.8	0.074	9.5		7.8	0.085	10.8			7.9	0.100	12.3		
3	8.1	0.136	18.2		8.3	0.087	12.8			8.4	0.121	15.1		
4	8.1	1.149	116.6		8.2	0.934	94.8			8.2	1.117	98.8		
5	7.9	3.153	268.0		7.7	2.968	265.5			7.8	2.580	212.9		
6	7.2	3.264	290.5		7.9	3.270	264.9			8.1	3.574	311.0		
7	8.1	3.519	299.1		8.1	3.434	291.9			8.2	3.754	309.7		
10	8.0	3.884	306.8	111.8	7.7	3.826	298.5	94.4		7.6	3.887	308.1	101.3	

^aDiluted with demineralized distilled water.^bCell counts with 10⁶/mL^cTotal cell volume (μ³ × 10⁶/mL)^dSuspended solids (mg/L)

APPENDIX C

TABLE C-IV. SECONDARY EFFLUENT ENRICHMENT OF CLEAR LAKE WATER

10% Secondary Effluent ^a					10% Secondary Effluent				10% Secondary Effluent			
Day	pH	CC ^b	TCV ^c	SS ^d	pH	CC	TCV	SS	pH	CC	TCV	SS
0		0.043	5.0			0.047	5.2			0.044	4.3	
2	8.0	0.040	6.1		8.0	0.032	4.8		7.9	0.045	6.9	
3	8.3	0.054	8.4		8.3	0.056	8.5		8.4	0.054	8.2	
4	8.1	1.177	120.6		8.1	1.330	139.0		8.1	1.100	103.3	
5	8.1	2.653	229.5		8.3	2.991	270.7		8.4	2.799	254.7	
6	8.4	3.888	316.9		8.2	3.767	325.8		8.2	3.821	326.7	
7	8.3	4.589	449.7		8.3	4.401	404.8		8.3	4.148	392.1	
10	7.9	4.528	352.1	129.1	7.5	4.517	356.8	146.1	7.5	4.382	346.0	116.1

20% Secondary Effluent					20% Secondary Effluent				20% Secondary Effluent			
Day	pH	CC	TCV	SS	pH	CC	TCV	SS	pH	CC	TCV	SS
0		0.046	5.2			0.039	4.6			0.045	4.9	
2	7.9	0.054	7.9		8.0	0.052	8.3		8.0	0.041	6.1	
3	8.3	0.064	10.4		8.4	0.059	10.1		8.5	0.047	7.4	
4	8.1	1.660	188.4		8.1	1.240	117.1		8.2	0.836	62.7	
5	8.3	2.490	252.2		8.4	3.049	309.5		8.2	0.824	82.4	
6	8.1	4.651	434.8		8.5	5.900	548.7		8.2	1.712	126.7	
7	8.4	6.216	618.5		8.4	7.213	692.5		8.4	1.364	137.1	
10	8.0	8.148	900.7	205.7	7.9	7.995	627.5	198.3	8.2	1.472	175.9	141.3

30% Secondary Effluent					30% Secondary Effluent				30% Secondary Effluent			
Day	pH	CC	TCV	SS	pH	CC	TCV	SS	pH	CC	TCV	SS
0		0.043	4.6			0.048	5.3			0.044	4.7	
2	7.9	0.063	9.4		7.8	0.065	9.6		7.8	0.059	8.8	
3	8.4	0.082	13.3		8.4	0.080	12.8		8.4	0.093	14.6	
4	8.2	1.466	157.6		8.1	1.475	150.4		8.1	1.326	130.6	
5	8.6	3.203	326.7		8.4	2.548	286.6		8.5	2.449	262.1	
6	8.3	6.096	542.5		8.5	5.274	527.4		8.5	6.122	547.8	
7	8.5	12.562	1249.9		8.6	10.853	1079.0		8.6	11.107	1089.6	
10	8.1	9.489	865.9	266.3	8.0	9.867	821.4	242.1	7.7	9.208	752.8	309.0

^aDiluted with Clear Lake water.^bCell counts x 10⁶/ml^cTotal cell volume (10⁶μ³/ml)^dSuspended solids (mg/l)

APPENDIX C

TABLE C-V. CLEAR LAKE ENRICHMENT (SPIKING) TESTS^a

Cl + P				Cl + Fe				Cl + Fe			
pH	CC	TCV	SS	pH	CC	TCV	SS	pH	CC	TCV	SS
8.3	0.366	39.6	87.6	8.3	0.345	36.2	97.2	8.3	0.199	20.3	100.0
	0.578	61.9			0.669	70.9			0.937	83.4	
	0.414	38.1			0.429	38.2			0.395		
	0.792	77.2			0.570	54.4			0.744	68.5	
	1.551	161.3			1.231	124.3			0.888	86.9	
	1.459	135.7			1.145	97.3			0.720	65.5	
	1.557	127.1			1.294	99.6					
	2.811	213.6			2.189	166.4			2.925	228.1	
Cl + N, P				Cl + N, P				Cl + N, Fe			
pH	CC	TCV	SS	pH	CC	TCV	SS	pH	CC	TCV	SS
8.4	0.340	35.7	100.0	8.3	0.273	26.5	122.0	8.3	0.238	23.1	191.5
	0.383	36.5			0.564	53.0			0.662	68.9	
	0.385	36.9			0.358	36.9			0.703	70.3	
	0.599	57.2			0.580	62.6			0.663	76.9	
	0.818	86.7			0.705	71.9			0.948	94.8	
	0.780	78.0			0.718	73.9			0.804	74.8	
	1.879	176.6			1.386	115.0			2.701	210.7	
	5.689	449.4			4.867	384.5			4.190	360.3	
Cl + N, Fe				Cl + P, Fe				Cl + P, Fe			
pH	CC	TCV	SS	pH	CC	TCV	SS	pH	CC	TCV	SS
8.3	0.308	35.1	122.4	8.3	0.320	33.9	100.0	8.3	0.284	29.5	104.0
	0.496	52.1			0.563	52.9			0.493	50.3	
	0.468	49.1			0.475	42.8			0.424	38.6	
	0.476	46.2			0.464	41.8			0.812	80.8	
	0.840	86.6			0.847	80.4			1.889	213.5	
	0.819	82.8			1.198	115.1			1.019	95.7	
	1.326	104.7			1.218	90.1			1.140	78.7	
	3.224	280.5			3.005	234.4			2.594	199.7	
Cl + N, P, Fe				Cl + N, P, Fe				Cl + N, P, Fe, Micron			
pH	CC	TCV	SS	pH	CC	TCV	SS	pH	CC	TCV	SS
8.3	0.315	34.3	144.2	8.3	0.285	30.2	126.5	8.4	0.301	31.9	202.5
	0.625	61.9			0.422	46.0			0.687	63.2	
	0.570	53.1			0.416	43.5			0.998	83.1	
	0.762	78.2			0.570	62.8			1.527	132.9	
	1.149	125.3			0.804	80.4			2.329	193.3	
	0.924	81.3			1.212	120.0			2.534	205.2	
	2.501	200.0			1.726	157.0			4.319	306.6	
	5.033	427.8			4.389	373.1			9.056	724.5	

TABLE C-V (Continued)

C1 + N, P, Fe, Micron.				C1 + 30% PAAP				C1 + 30% PAAP			
pH	CC	TCV	SS	pH	CC	TCV	SS	pH	CC	TCV	SS
8.4	0.351	42.1		8.5	0.328	38.0		8.5	0.319	36.7	
	0.815	76.6			1.034	109.6			0.865	88.3	
	1.237	116.5			0.906	83.4			1.249	138.6	
	2.061	206.1			2.128	195.8			1.717	195.4	
	2.678	227.6			2.150	195.6			2.295	257.1	
	3.099	266.5			3.310	284.7			2.089	206.0	
	5.067	476.3			6.705	509.5			6.248	556.0	
	13.100	1558.0	212.5		12.604	1499.8	300.0		15.759	2096.0	310.4
100% C1				100% C1				100% C1			
pH	CC	TCV	SS	pH	CC	TCV	SS	pH	CC	TCV	SS
8.3	0.301	32.5		8.3	0.320	34.9		8.3	0.283	30.3	
	0.358	36.2			0.381	41.2			0.421	42.5	
	0.506	53.4			0.402	43.2			0.567	59.5	
	0.118	12.1			0.466	45.5			0.485	49.2	
	0.826	83.4			0.819	81.1			0.936	95.4	
	1.174	108.0			1.109	108.7			0.964	98.3	
	2.000	192.0			1.211	104.1			3.104	310.4	
	2.370	236.1	160.0		2.732	225.4	108.0		2.466	204.7	94.8
C1 + 10% SE				C1 + 10% SE				C1 + 10% SE			
pH	CC	TCV	SS	pH	CC	TCV	SS	pH	CC	TCV	SS
8.3	0.267	29.9		8.3	0.290	32.2		8.3	0.297	34.2	
	0.419	45.7			0.464	53.1			0.422	44.7	
	0.394	42.3			0.411	43.1			0.509	50.4	
	0.602	63.5			0.464	51.5			0.632	67.4	
	0.737	78.1			0.547	62.3			1.155	135.1	
	0.936	111.4			1.043	119.9			1.285	142.7	
	2.552	244.9			1.646	182.7			2.205	194.1	
	5.220	433.3	127.4		4.572	420.6	124.5		5.784	497.4	164.7
C1 + N				C1 + N				C1 + P			
pH	CC	TCV	SS	pH	CC	TCV	SS	pH	CC	TCV	SS
8.3	0.343	36.9		8.3	0.314	32.1		8.3	0.334	35.4	
	0.400	42.4			0.426	42.6			0.380	38.6	
	0.415	41.9			0.297	31.7			0.490	45.8	
	0.706	72.4			0.480	50.2			0.893	99.6	
	0.832	91.6			1.123	131.4			1.654	168.7	
	1.204	119.2			1.022	122.6			0.890	83.7	
	2.410	226.5			1.044	93.9			2.268	199.6	
	3.371	330.4	140.0		2.754	250.6	92.3		2.472	187.9	98.7

^aMeasurements made on days 2, 3, 4, 5, 6, 7, 10, 16, respectively.

APPENDIX C

TABLE C-VI. SACRAMENTO RIVER STUDY

100% SR ^a					100% SR				100% SR			
Day	pH	CC ^b	TCVC	SS ^d	pH	CC	TCV	SS	pH	CC	TCV	SS
0		0.030	3.2			0.034	3.6			0.034	3.3	
3	8.0	0.065	8.4		8.9	0.074	9.1		7.9	0.074	8.4	
4		0.212	16.8			0.213	20.9			0.298	25.4	
6		0.238	19.9			0.247	21.5			0.260	18.5	
7		0.276	20.7			0.274	23.8			0.301	23.5	
10		0.363	26.5			0.284	23.6			0.283	22.3	
14		0.389	32.3	15.6		0.311	25.7	17.1		0.301	21.7	13.0

66.7% SR					66.7% SR				66.7% SR			
Day	pH	CC	TCV	SS	pH	CC	TCV	SS	pH	CC	TCV	SS
0		0.042	2.8			0.025	2.1			0.026	2.3	
3	7.9	0.064	6.3		7.9	0.102	8.6		8.3	0.115	9.7	
4		0.192	18.4			0.222	19.6			0.179	12.7	
6		0.193	13.6			0.195	12.9			0.208	14.5	
7		0.214	15.1			0.216	15.3			0.215	15.0	
10		0.258	18.6			0.256	18.8			0.262	18.4	
14		0.307	24.6	10.0		0.229	19.8	10.3		0.237	15.6	10.8

33.3% SR					33.3% SR				33.3% SR			
Day	pH	CC	TCV	SS	pH	CC	TCV	SS	pH	CC	TCV	SS
0		0.019	1.2			0.018	1.1			0.019	1.7	
3	7.5	0.067	4.7		7.6	0.065	4.7		7.5	0.069	5.2	
4		0.087	6.6			0.087	7.1			0.085	6.1	
6		0.092	6.0			0.094	6.4			0.095	6.4	
7		0.103	6.8			0.105	7.1			0.107	7.5	
10		0.113	7.9			0.111	7.4			0.123	8.6	
14		0.113	7.8	6.0		0.130	9.0	5.8		0.159	11.3	5.7

30% PAAP Control					30% PAAP Control				30% PAAP Control			
Day	pH	CC	TCV	SS	pH	CC	TCV	SS	pH	CC	TCV	SS
0		0.022	1.6			0.020	1.3			0.022	1.4	
3	8.4	0.075	8.4		7.1	0.081	8.3		6.9	0.061	6.2	
4		0.378	39.3			0.301	33.5			0.238	22.2	
6		3.882	287.2			2.031	204.0			2.895	266.4	
7	8.6	4.848	360.6		8.2	3.232	302.2		8.1	5.532	409.4	
10		5.936	463.0			3.945	374.8			7.447	536.2	
14		6.649	558.5	157.6		3.810	382.9	139.9		5.296	389.2	159.1

SR + 30% PAAP					SR + 30% PAAP				SR + 30% PAAP			
Day	pH	CC	TCV	SS	pH	CC	TCV	SS	pH	CC	TCV	SS
0		0.026	1.9			0.060	6.1			0.030	3.1	
3	7.0	0.040	5.6		7.4	0.054	8.4		7.5	0.071	11.6	
4		1.959	25.3			0.268	32.4			0.197	24.2	
6		2.330	188.8			5.197	462.5			2.797	243.4	
7	8.1	2.773	196.8		8.0	2.927	209.3		8.1	4.098	293.0	
10		4.214	598.4			3.703	277.7			3.654	358.0	
14		4.942	375.6	70.7		3.933	298.9	64.0		4.408	335.0	70.0

^aSacramento River water.^bCell counts x 10⁶/ml^cTotal cell volume (10⁶ μ³/ml)^dSuspended Solids (mg/l)

APPENDIX C

TABLE C-VII. SECONDARY EFFLUENT ENRICHMENT OF SACRAMENTO RIVER WATER

+10% SE ^a					+10% SE				+10% SE			
Day	pH	CC ^b	TCVC	SS ^d	pH	CC	TCV	SS	pH	CC	TCV	SS
0		0.025	2.1			0.027	2.2			0.029	2.7	
3	8.1	0.073	11.4		7.8	0.059	9.0		8.0	0.051	7.3	
4		0.422	50.6			0.244	31.3			0.229	35.5	
6		2.804	243.3			2.700	199.8			3.302	174.0	
7	8.5	3.773	317.0		8.5	3.878	312.2		8.5	4.382	339.6	
10		3.648	277.2			3.562	277.8			5.621	455.3	
14		5.285	422.8	73.1		8.901	716.5	91.5		7.198	529.0	115.8

+20% SE					+20% SE				+20% SE			
Day	pH	CC	TCV	SS	pH	CC	TCV	SS	pH	CC	TCV	SS
0		0.026	2.6			0.035	3.7			0.030	2.8	
3	7.8	0.088	14.8		8.5	0.097	14.9		7.3	0.080	9.9	
4		0.383	59.8			0.332	49.4			0.297	38.3	
6		5.140	439.5			3.717	306.7			3.499	265.9	
7	8.5	6.218	500.6		8.5	5.050	414.1		8.5	4.942	390.1	
10		8.264	673.5			5.444	454.6			4.947	405.7	
14		7.477	568.2	165.2		8.087	622.7	131.9		5.518	444.2	134.5

+30% SE					+30% SE				+30% SE			
Day	pH	CC	TCV	SS	pH	CC	TCV	SS	pH	CC	TCV	SS
0		0.042	4.4			0.035	3.4			0.038	4.3	
3	7.6	0.060	9.7		7.8	0.063	6.0		8.2	0.061	9.8	
4		0.288	48.1			0.209	34.1			0.195	30.8	
6		5.204	463.4			2.853	286.7			3.452	329.6	
7	8.6	8.067	705.8		8.6	6.282	559.1		8.2	6.463	568.8	
10		10.146	842.1			9.766	942.4			8.814	696.3	
14		14.428	1154.2	247.0		13.675	1053.0	221.5		13.823	1089.9	210.4

^aSecondary Effluent.

^bCell counts $\times 10^6/\text{ml}$

^cTotal cell volume ($10^6 \mu^3/\text{ml}$)

^dSuspended solids (mg/l)

APPENDIX C

TABLE C-VIII. SACRAMENTO RIVER ENRICHMENT (SPIKING) TESTS

100% SR					100% SR					100% SR				
Day	pH	CC	TCV	SS	pH	CC	TCV	SS		pH	CC	TCV	SS	
0	8.45	0.099	12.3		8.45	0.099	12.4			8.45	0.103	12.7		
2		0.128	14.0			0.065	7.9				0.097	10.9		
3	8.45	0.116	11.9		8.35	0.139	14.6			8.45	0.147	16.9		
4	8.2	0.266	27.4		8.3	0.243	23.6			8.2	0.230	23.5		
5		0.171	16.8			0.193	18.5				0.204	20.8		
6		0.215	20.5			0.238	22.9				0.272	26.1		
7		0.255	22.8			0.250	21.7				0.259	22.6		
10		0.942	65.2	32.8		0.860	60.2	30.4			0.955	71.7	31.7	
SR + 10% SE					SR + 10% SE					SR + 10% SE				
Day	pH	CC	TCV	SS	pH	CC	TCV	SS		pH	CC	TCV	SS	
0	8.4	0.108	13.3		8.4	0.129	15.5			8.4	0.141	17.0		
2		0.068	9.3			0.092	12.1				0.072	9.9		
3	8.35	0.146	20.0		8.55	0.142	19.3			8.55	0.139	20.4		
4	8.5	0.261	31.4		8.3	0.188	25.7			8.4	0.209	27.6		
5		0.229	27.5			0.226	29.2				0.254	24.6		
6		0.262	30.2			0.322	39.6				0.286	35.5		
7		0.962	75.5			1.217	124.1				1.465	161.9		
10		4.189	332.0	73.6		4.131	313.9	72.2			1.962	151.1	80.0	
SR + N					SR + N					SR + P				
Day	pH	CC	TCV	SS	pH	CC	TCV	SS		pH	CC	TCV	SS	
0	8.45	0.090	11.2		8.45	0.083	11.1			8.45	0.084	11.1		
2		0.116	13.3			0.111	12.8				0.107	12.4		
3	8.4	0.148	15.9		8.6	0.156	16.8			8.6	0.168	17.5		
4	8.4	0.163	16.6		8.4	0.151	15.3			8.4	0.183	18.5		
5		0.189	18.3			0.155	13.9				0.209	20.2		
6		0.209	19.9			0.231	21.2				0.249	23.0		
7		0.242	21.0			0.247	21.7				0.304	28.5		
10		1.391	114.8	43.1		4.450	318.2	45.1			1.564	122.4	58.7	
SR + P					SR + Fe					SR + Fe				
Day	pH	CC	TCV	SS	pH	CC	TCV	SS		pH	CC	TCV	SS	
0	8.45	0.106	11.8		8.45	0.080	9.4			8.45	0.154	14.3		
2		0.110	12.4			0.138	15.3				0.146	15.3		
3	8.5	0.160	15.4		8.55	0.149	14.6			8.35	0.174	17.9		
4	8.4	0.152	14.3		8.4	0.162	15.0			8.4	0.152	14.3		
5		0.175	16.9			0.939	77.4				0.305	107.7		
6		0.293	28.6			1.149	92.5				0.447	38.9		
7		0.271	23.6			0.830	63.5				0.963	73.2		
10		0.984	75.5	37.8		0.997	74.8	34.1			1.063	77.6	32.3	
SR + N, P					SR + N, P					SR + N, Fe				
Day	pH	CC	TCV	SS	pH	CC	TCV	SS		pH	CC	TCV	SS	
0	8.45	0.078	9.1		8.45	0.130	13.3			8.45	0.093	11.3		
2		0.105	12.2			0.126	12.6				0.133	14.7		
3	8.5	0.220	20.8		8.45	0.262	24.7			8.45	0.202	20.4		
4	8.5	0.229	20.4		8.4	0.265	22.8			8.4	0.289	28.3		
5		1.083	91.5			1.302	113.9				1.341	104.6		
6		1.382	106.4			2.247	174.1				1.636	111.2		
7		1.960	149.0			3.285	236.5				4.889	364.0		
10		7.602	447.0	111.6		7.861	495.2	110.5			2.200	184.8	68.6	

TABLE C-VIII (Continued)

SR + N, Fe					SR + P, Fe					SR + P, Fe				
Day	pH	CC	TCV	SS	pH	CC	TCV	SS		pH	CC	TCV	SS	
0	8.45	0.092	11.3		8.45	0.100	11.9			8.45	0.066	7.6		
2		0.112	12.6			0.099	11.2				0.097	10.4		
3	8.45	0.218	21.9		8.5	0.193	17.9			8.45	0.196	18.8		
4	8.7	0.259	25.4		8.3	0.274	25.5			8.5	0.253	21.7		
5		1.155	97.6			0.850	67.5				2.755	181.8		
6		1.576	110.3			1.231	82.5				1.414	131.6		
7		1.374	93.4			1.387	101.2				2.408	169.8		
10		1.674	146.1	54.3		1.044	77.2	35.6			1.012	79.7	33.3	

SR + N, P, Fe					SR + N, P, Fe					SR + N, P, Fe, Micron.				
Day	pH	CC	TCV	SS	pH	CC	TCV	SS		pH	CC	TCV	SS	
0	8.45	0.088	11.0		8.0	0.076	9.4			8.45	0.093	11.2		
2		0.113	12.4			0.124	13.5				0.160	18.1		
3	8.4	0.196	18.8		8.5	0.222	21.8			8.6	0.496	47.9		
4	8.4	0.237	22.3		8.4	0.279	26.2			8.7	4.961	352.2		
5		0.983	87.0			1.061	91.8				6.516	436.6		
6		1.594	121.9			1.732	129.9				6.886	454.5		
7		1.844	131.8			2.634	183.1				6.262	388.3		
10		7.671	487.1	119.5		8.102	514.5	110.6			8.476	529.7	126.2	

SR + N, P, Fe, Micron.					SR + 30 PAAP					SR + 30 PAAP				
Day	pH	CC	TCV	SS	pH	CC	TCV	SS		pH	CC	TCV	SS	
0	8.45	0.115	11.9		8.0	0.060	4.1			8.1	0.087	8.4		
2		0.142	15.8			0.084	8.6				0.144	15.8		
3	8.25	0.441	45.5		8.35	0.229	21.6			8.35	0.357	36.2		
4	8.8	2.528	203.5		8.7	2.430	240.5			8.7	1.913	156.9		
5		5.330	378.4			4.822	361.6				2.856	217.0		
6		7.694	480.8			7.234	491.8				6.087	404.8		
7		8.249	519.7			8.223	542.7				7.463	548.5		
10		9.036	560.2	150.0		10.084	675.6	182.6			10.240	701.4	182.6	

30 PAAP				
Day	pH	CC	TCV	SS
0	7.6	0.019	1.4	
2		0.049	4.3	
3	7.85	0.206	19.5	
4	8.1	1.876	186.7	
5		4.594	337.5	
6		8.694	652.0	
7		10.908	823.6	
10		10.304	795.4	164.5

APPENDIX D
CHEMOSTAT OPERATION DATA

Chemostat Number 1; Chemostat Volume 1,040 ml

Time day	Residence Time days	S ₀ PO ₄ $\mu\text{g/l-P}$	pH Units	Absorbance OD/inch	Time day	Residence Time days	S ₀ PO ₄ $\mu\text{g/l-P}$	pH Units	Absorbance OD/inch
1	-	-	-	-	28	0.951	64.8	7.0	0.045
2	-	-	-	-	29	0.951	64.8	7.0	0.040
3	-	-	-	-	30	0.951	64.8	6.9	0.037
4	1.39	100	7.9	0.136	31 ^a	0.951	64.8	6.9	0.040
5	1.39	100	8.2	0.120	32	0.902	64.8	7.3	0.037
6	1.39	100	8.6	0.087	33	0.902	64.8	7.6	0.040
7	1.39	100	9.3	0.078	34 ^a	0.902	64.8	7.5	0.040
8 ^a	1.39	100	7.4	0.065	35	0.756	66.7	-	-
9	1.37	100	-	-	36	0.756	66.7	6.9	0.040
10	1.37	100	-	-	37	0.756	66.7	6.9	0.038
11	1.37	100	7.7	0.073	38	0.756	66.7	6.9	0.036
12	1.37	63	-	-	39	0.756	66.7	6.9	0.036
13	1.37	63	-	-	40	4.89	65.1	-	-
14	1.37	63	7.9	0.036	41	4.89	65.1	8.3	0.096
15	1.37	63	8.0	0.030	42	4.89	65.1	8.4	0.112
16 ^a	1.37	63	8.0	0.040	43	4.89	65.1	8.4	0.107
17	3.04	67.5	8.7	0.050	44	4.89	65.1	8.0	0.106
18	3.04	67.5	8.7	0.053	45 ^a	4.89	65.1	8.3	0.100
19	3.04	67.5	-	-	46	4.78	65.1	8.0	0.096
20	3.04	67.5	7.9	0.053	47 ^a	4.78	65.1	8.3	0.094
21	3.04	67.5	7.0	0.048	48	Non-flow system	65.1	-	-
22	2.91	67.5	7.3	0.045	49	Non-flow system	65.1	-	-
23	2.91	67.5	7.3	0.050	50	Non-flow system	65.1	-	-
24	2.91	67.5	7.2	0.050	51	Non-flow system	65.1	8.0	0.120
25	2.91	67.5	7.2	0.055	52	Non-flow system	65.1	7.9	0.125
26 ^a	2.91	67.5	7.7	0.050	53 ^a	Non-flow system	65.1	8.0	0.120
27	0.951	64.8	-	-	54	Non-flow system	65.1	8.3	0.125
					55 ^a	Non-flow system	65.1	8.6	0.120

Chemostat Number 2; Chemostat Volume 1,040 ml

Time day	Residence Time days	S ₀ PO ₄ $\mu\text{g/l-P}$	pH Units	Absorbance OD/inch	Time day	Residence Time days	S ₀ PO ₄ $\mu\text{g/l-P}$	pH Units	Absorbance OD/inch
1	-	-	-	-	28	0.940	63.3	7.0	0.045
2	-	-	-	-	29	0.940	63.3	7.0	0.040
3	-	-	-	-	30	0.940	63.3	7.0	0.030
4	1.39	100	8.0	0.147	31 ^a	0.940	63.3	7.0	0.037
5	1.39	100	8.2	0.120	32	0.880	63.3	7.5	0.037
6	1.39	100	8.1	0.080	33	0.880	63.3	7.6	0.042
7	1.39	100	8.3	0.070	34 ^a	0.880	63.3	7.6	0.040
8 ^a	1.39	100	7.9	0.065	35	0.527 ^b	66.7	-	-
9	1.32	100	-	-	36	0.527 ^b	66.7	6.8	0.016
10	1.32	100	-	-	37	0.527 ^b	66.7	7.3	0.010
11	1.32	100	8.0	0.064	38	0.527 ^b	66.7	6.8	0.006
12	1.32	62	-	-	39	0.527 ^b	66.7	6.8	0.005
13	1.32	62	-	-	40	4.69	69.0	-	-
14	1.32	62	7.9	0.040	41	4.69	69.0	8.3	0.054
15	1.32	62	7.8	0.036	42	4.69	69.0	8.4	0.112
16 ^a	1.32	62	8.0	0.040	43	4.69	69.0	8.4	0.114
17	2.87	68.7	8.4	0.052	44	4.69	69.0	7.9	0.113
18	2.87	68.7	8.6	0.055	45 ^a	4.69	69.0	8.2	0.104
19	2.87	68.7	-	-	46	4.78	69.0	8.0	0.097
20	2.87	68.7	7.9	0.057	47 ^a	4.78	69.0	8.2	0.095
21 ^a	2.87	68.7	7.4	0.052	48	Non-flow system	69.0	-	-
22	2.86	68.7	7.5	0.045	49	Non-flow system	69.0	-	-
23	2.86	68.7	7.3	0.055	50	Non-flow system	69.0	-	-
24	2.86	68.7	7.1	0.057	51	Non-flow system	69.0	7.9	0.130
25	2.86	68.7	7.3	0.051	52	Non-flow system	69.0	8.0	0.130
26 ^a	2.86	68.7	7.3	0.051	53 ^a	Non-flow system	69.0	8.2	0.125
27	0.940	63.3	-	-	54	Non-flow system	69.0	8.3	0.125
					55	Non-flow system	69.0	8.6	0.120

^aSampling days

^b"Wash-out" occurs.

CHEMOSTAT OPERATION DATA (Continued)

Chemostat Number 3; Chemostat Volume 1,020 ml

Time day	Residence Time days	S_{OpP} $\mu\text{g/l-P}$	pH Units	Absorbance OD/inch	Time day	Residence Time days	S_{OpP} $\mu\text{g/l-P}$	pH Units	Absorbance OD/inch
1	-	-	-	-	28	0.823	63.3	7.0	0.040
2	-	-	-	-	29	0.823	63.3	7.1	0.030
3	-	-	-	-	30	0.823	63.3	7.0	0.030
4	1.78	103	8.0	0.135	31 ^a	0.823	63.3	7.0	0.040
5	1.78	103	8.1	0.109	32	0.773	63.3	7.6	0.040
6	1.78	103	8.2	0.080	33	0.773	63.3	7.6	0.045
7	1.78	103	8.3	0.065	34 ^a	0.773	63.3	7.6	0.042
8 ^a	1.78	103	7.9	0.060	35	0.737	66.7	-	-
9	1.13	103	-	-	36	0.737	66.7	6.8	0.037
10	1.13	103	-	-	37	0.737	66.7	7.7	0.036
11	1.13	64	8.1	0.085	38	0.737	66.7	6.8	0.040
12	1.13	64	-	-	39	0.737	66.7	6.8	0.038
13	1.13	64	-	-	40	4.80	66.3	-	-
14	1.13	64	8.0	0.030	41	4.80	66.3	8.3	0.090
15	1.13	64	7.8	0.030	42	4.80	66.3	8.3	0.100
16 ^a	1.13	64	7.9	0.036	43	4.80	66.3	8.4	0.114
17	2.61	69.3	8.3	0.043	44	4.80	66.3	8.0	0.100
18	2.61	69.3	8.6	0.053	45 ^a	4.80	66.3	7.9	0.100
19	2.61	69.3	-	-	46	4.69	66.3	7.8	0.096
20	2.61	69.3	7.8	0.041	47 ^a	4.69	66.3	8.2	0.093
21 ^a	2.61	69.3	7.2	0.037	48	Non-flow	66.3	-	-
22	2.64	69.3	7.5	0.035	49	system	66.3	-	-
23	2.64	69.3	7.3	0.043	50		66.3	-	-
24	2.64	69.3	7.0	0.047	51		66.3	7.3	0.125
25	2.64	69.3	7.3	0.055	52		66.3	7.1	0.120
26 ^a	2.64	69.3	7.8	0.053	53 ^a		66.3	8.0	0.120
27	0.823	63.3	-	-	54		66.3	8.3	0.125
					55 ^a		66.3	8.4	0.120

Chemostat Number 4; Chemostat Volume 1,020 ml

Time day	Residence Time days	S_{OpP} $\mu\text{g/l-P}$	pH Units	Absorbance OD/inch	Time day	Residence Time days	S_{OpP} $\mu\text{g/l-P}$	pH Units	Absorbance OD/inch
1	-	-	-	-	28	0.955	100	7.0	0.065
2	-	-	-	-	29	0.955	100	7.1	0.050
3	-	-	-	-	30	0.955	100	7.1	0.042
4	1.37	116	8.1	0.203	31 ^a	0.955	100	7.1	0.050
5	1.37	116	8.3	0.185	32	0.905	100	7.6	0.057
6	1.37	116	8.3	0.110	33	0.905	100	7.7	0.062
7	1.37	116	8.0	0.083	34 ^a	0.905	100	7.7	0.060
8 ^a	1.37	116	7.9	0.075	35	0.556	100	-	-
9	1.29	116	-	-	36	0.556	100	6.9	0.060
10	1.29	116	-	-	37	0.556	100	7.7	0.057
11	1.29	116	8.1	0.065	38	0.556	100	7.0	0.060
12	1.29	99	-	-	39	0.556	100	6.9	0.060
13	1.29	99	-	-	40	4.82	103	-	-
14	1.29	99	8.2	0.045	41	4.82	103	8.3	0.140
15	1.29	99	7.9	0.045	42	4.82	103	8.4	0.190
16 ^a	1.29	99	7.9	0.050	43	4.82	103	8.4	0.185
17	3.06	104	8.3	0.067	44	4.82	103	7.6	0.183
18	3.06	104	8.4	0.077	45 ^a	4.82	103	7.9	0.180
19	3.06	104	-	-	46	4.82	103	7.5	0.180
20	3.06	104	7.9	0.080	47 ^a	4.82	103	8.0	0.175
21 ^a	3.06	104	7.2	0.078	48	Non-flow	103	-	-
22	2.87	104	7.5	0.060	49	system	103	-	-
23	2.87	104	7.3	0.065	50		103	-	-
24	2.87	104	7.0	0.067	51		103	7.1	0.210
25	2.87	104	7.4	0.080	52		103	6.9	0.213
26 ^a	2.87	104	7.8	0.080	53 ^a		103	7.5	0.215
27	0.955	100	-	-	54		103	8.3	0.220
					55 ^a		103	8.5	0.220

^aSampling days

CHEMOSTAT OPERATION DATA (Continued)
Chemostat Number 5; Chemostat Volume 1,070 ml

Time day	Residence Time days	S _{opp} , μg/l-P	pH Units	Absorbance OD/inch	Time day	Residence Time days	S _{opp} , μg/l-P	pH Units	Absorbance OD/inch
1	-	-	-	-	28	0.967	93.3	7.1	0.060
2	-	-	-	-	29	0.967	93.3	7.2	0.047
3	-	-	-	-	30	0.967	93.3	7.1	0.045
4	1.43	120	8.0	0.165	31 ^a	0.967	93.3	7.2	0.052
5	1.43	120	8.3	0.141	32	0.861	93.3	7.6	0.050
6	1.43	120	8.2	0.100	33	0.861	93.3	7.7	0.052
7	1.43	120	8.3	0.085	34 ^a	0.861	93.3	7.7	0.053
8 ^a	1.43	120	8.0	0.081	35	0.740	100	-	-
9	1.36	120	-	-	36	0.740	100	6.9	0.056
10	1.36	120	-	-	37	0.740	100	7.7	0.058
11	1.36	120	8.0	0.075	38	0.740	100	6.8	0.055
12	1.36	99	-	-	39	0.740	100	6.9	0.058
13	1.36	99	-	-	40	5.10	106	-	-
14	1.36	99	8.3	0.063	41	5.10	106	8.3	0.162
15	1.36	99	7.9	0.064	42	5.10	106	8.4	0.194
16 ^a	1.36	99	7.8	0.060	43	5.10	106	8.4	0.198
17	3.06	104	8.6	0.085	44	5.10	106	7.7	0.183
18	3.06	104	8.2	0.088	45 ^a	5.10	106	8.0	0.184
19	3.06	104	-	-	46	5.03	106	7.4	0.175
20	3.06	104	7.7	0.085	47 ^a	5.03	106	8.1	0.180
21 ^a	3.06	104	7.2	0.088	48	Non-flow	106	-	-
22	2.86	104	7.5	0.070	49	system	106	-	-
23	2.86	104	7.3	0.077	50	↓	106	-	-
24	2.86	104	7.4	0.076	51	↓	106	7.2	0.220
25	2.86	104	7.4	0.085	52	↓	106	7.1	0.220
26 ^a	2.86	104	7.8	0.080	53 ^a	↓	106	7.6	0.215
27	0.967	93.3	-	-	54	↓	106	8.3	0.220
					55 ^a	↓	106	8.3	0.210

Chemostat Number 6; Chemostat Volume 1,080 ml

1	-	-	-	-	28	0.910	96.3	7.1	0.056
2	-	-	-	-	29	0.910	96.3	7.3	0.045
3	-	-	-	-	30	0.910	96.3	7.2	0.048
4	1.38	117	8.1	0.135	31 ^a	0.910	96.3	7.2	0.049
5	1.38	117	8.2	0.115	32	0.869	96.3	7.6	0.054
6	1.38	117	8.3	0.089	33	0.869	96.3	7.7	0.056
7	1.38	117	8.4	0.085	34 ^a	0.869	96.3	7.7	0.055
8 ^a	1.38	117	8.2	0.083	35	0.668	100	-	-
9	1.19	117	-	-	36	0.668	100	6.8	0.056
10	1.19	117	-	-	37	0.668	100	7.7	0.056
11	1.19	117	8.0	0.066	38	0.668	100	6.9	0.060
12	1.19	94	-	-	39	0.668	100	6.9	0.057
13	1.19	94	-	-	40	5.08	101	-	-
14	1.19	94	8.3	0.050	41	5.08	101	8.3	0.140
15	1.19	94	8.0	0.053	42	5.08	101	8.3	0.194
16 ^a	1.19	94	7.9	0.060	43	5.08	101	8.4	0.195
17	2.69	100	8.0	0.085	44	5.08	101	7.5	0.188
18	2.69	100	7.9	0.095	45 ^a	5.08	101	8.0	0.190
19	2.69	100	-	-	46	4.97	101	7.4	0.180
20	2.69	100	7.7	0.085	47 ^a	4.97	101	8.0	0.180
21 ^a	2.69	100	7.2	0.088	48	Non-flow	101	-	-
22	2.74	100	7.5	0.065	49	system	101	-	-
23	2.74	100	7.3	0.070	50	↓	101	-	-
24	2.74	100	7.0	0.073	51	↓	101	7.0	0.235
25	2.74	100	7.4	0.085	52	↓	101	7.0	0.230
26 ^a	2.74	100	7.9	0.075	53 ^a	↓	101	7.9	0.230
27	0.910	96.3	-	-	54	↓	101	8.3	0.230
					55 ^a	↓	101	8.5	0.235

^aSampling days.

CHEMOSTAT OPERATION DATA (Continued)

Chemostat Number 7; Chemostat Volume 1,050 ml

Time day	Residence Time days	S _{OPOL} g/l-P	pH Units	Absorbance OD/inch	Time day	Residence Time days	S _{OPOL} g/l-P	pH Units	Absorbance OD/inch
1	-	-	-	-	28	0.960	128	7.2	0.085
2	-	-	-	-	29	0.960	128	7.3	0.075
3	-	-	-	-	30	0.960	128	7.2	0.070
4	1.48	136	8.3	0.165	31 ^a	0.960	128	7.2	0.080
5	1.48	136	8.2	0.148	32	0.906	128	7.6	0.072
6	1.48	136	8.3	0.118	33	0.906	128	7.9	0.070
7	1.48	136	8.6	0.110	34 ^a	0.906	128	7.9	0.075
8 ^a	1.48	136	8.4	0.106	35	0.726	138	-	-
9	1.27	136	-	-	36	0.726	138	7.0	0.069
10	1.27	136	-	-	37	0.726	138	7.8	0.070
11	1.27	136	8.2	0.095	38	0.726	138	7.0	0.073
12	1.27	129	-	-	39	0.726	138	7.0	0.075
13	1.27	129	-	-	40	5.08	137	-	-
14	1.27	129	8.3	0.066	41	5.08	137	8.3	0.195
15	1.27	129	7.9	0.070	42	5.08	137	8.4	0.258
16 ^a	1.27	129	7.9	0.077	43	5.08	137	8.4	0.256
17	2.74	135	8.1	0.110	44	5.08	137	7.6	0.240
18	2.74	135	8.0	0.130	45 ^a	5.08	137	8.0	0.247
19	2.74	135	-	-	46	4.93	137	7.4	0.233
20	2.74	135	7.6	0.130	47 ^a	4.93	137	8.0	0.223
21 ^a	2.74	135	7.3	0.125	48	Non-flow system ↓	137	-	-
22	2.88	135	7.5	0.116	49		137	-	-
23	2.88	135	7.3	0.127	50		137	-	-
24	2.88	135	7.1	0.120	51		137	7.0	0.300
25	2.88	135	7.5	0.140	52		137	7.0	0.300
26 ^a	2.88	135	7.9	0.135	53 ^a		137	7.9	0.290
27	0.960	128	-	-	54		137	8.3	0.295
					55 ^a		137	8.5	0.286

Chemostat Number 8; Chemostat Volume 1,030 ml

Time day	Residence Time days	S _{OPOL} g/l-P	pH Units	Absorbance OD/inch	Time day	Residence Time days	S _{OPOL} g/l-P	pH Units	Absorbance OD/inch
1	-	-	-	-	28	0.873	130	7.3	0.085
2	-	-	-	-	29	0.873	130	7.3	0.075
3	-	-	-	-	30	0.873	130	7.3	0.080
4	1.45	136	8.2	0.165	31 ^a	0.873	130	7.3	0.085
5	1.45	136	8.2	0.140	32	0.876	130	7.7	0.075
6	1.45	136	8.4	0.110	33	0.876	130	7.9	0.075
7	1.45	136	8.7	0.099	34 ^a	0.876	130	8.0	0.078
8 ^a	1.45	136	8.4	0.090	35	0.655	138	-	-
9	1.20	136	-	-	36	0.655	138	7.0	0.070
10	1.20	136	-	-	37	0.655	138	7.9	0.065
11	1.20	136	8.1	0.075	38	0.655	138	7.2	0.067
12	1.20	130	-	-	39	0.655	138	7.0	0.070
13	1.20	130	-	-	40	4.58	137	-	-
14	1.20	130	8.3	0.083	41	4.58	137	8.3	0.170
15	1.20	130	8.0	0.083	42	4.58	137	8.5	0.220
16 ^a	1.20	130	8.0	0.080	43	4.58	137	8.4	0.230
17	2.69	136	8.8	0.126	44	4.58	137	7.5	0.225
18	2.69	136	7.8	0.147	45 ^a	4.58	137	7.9	0.228
19	2.69	136	-	-	46	4.55	137	7.4	0.225
20	2.69	136	7.6	0.140	47 ^a	4.55	137	8.0	0.223
21 ^a	2.69	136	7.4	0.140	48	Non-flow system ↓	137	-	-
22	2.68	136	7.5	0.127	49		137	-	-
23	2.68	136	7.3	0.120	50		137	-	-
24	2.68	136	7.1	0.126	51		137	7.2	0.280
25	2.68	136	7.5	0.135	52		137	7.5	0.275
26 ^a	2.68	136	8.3	0.130	53 ^a		137	7.9	0.280
27	0.873	130	-	-	54		137	8.3	0.280
					55 ^a		137	8.5	0.280

^aSampling days.

CHEMOSTAT OPERATION DATA (Continued)

Chemostat Number 9; Chemostat Volume 1,055 ml.

Time day	Residence Time days	S ₀ PO ₄ μ g/l-P	pH Units	Absorbance OD/inch	Time day	Residence Time days	S ₀ PO ₄ μ g/l-P	pH Units	Absorbance OD/inch
1	-	-	-	-	28	0.870	128	7.3	0.087
2	-	-	-	-	29	0.870	128	7.3	0.075
3	-	-	-	-	30	0.870	128	7.3	0.075
4	1.30	137	8.1	0.073	31 ^a	0.870	128	7.3	0.078
5	1.30	137	8.4	0.163	32	0.829	128	7.7	0.070
6	1.30	137	8.3	0.093	33	0.829	128	8.0	0.065
7	1.30	137	8.5	0.099	34 ^a	0.829	128	8.0	0.070
8 ^a	1.30	137	8.4	0.095	35	0.739	138	-	-
9	1.25	137	-	-	36	0.739	138	6.9	0.057
10	1.25	137	-	-	37	0.739	138	7.8	0.061
11	1.25	137	8.1	0.065	38	0.739	138	6.9	0.068
12	1.25	129	-	-	39	0.739	138	7.0	0.065
13	1.25	129	-	-	40	4.89	136	-	-
14	1.25	129	8.3	0.081	41	4.89	136	8.3	0.170
15	1.25	129	8.0	0.076	42	4.89	136	8.3	0.230
16 ^a	1.25	129	7.9	0.070	43	4.89	136	8.4	0.250
17	2.68	133	8.7	0.105	44	4.89	136	7.5	0.245
18	2.68	133	7.8	0.127	45 ^a	4.89	136	8.0	0.240
19	2.68	133	-	-	46	4.85	136	7.4	0.233
20	2.68	133	7.5	0.135	47 ^a	4.85	136	8.0	0.235
21 ^a	2.68	133	7.3	0.135	48	Non-flow system	136	-	-
22	2.63	133	7.5	0.117	49	Non-flow system	136	-	-
23	2.63	133	7.3	0.117	50	Non-flow system	136	-	-
24	2.63	133	7.1	0.123	51	Non-flow system	136	7.4	0.285
25	2.63	133	7.5	0.136	52	Non-flow system	136	7.2	0.290
26 ^a	2.63	133	8.0	0.140	53 ^a	Non-flow system	136	7.8	0.290
27	0.870	128	-	-	54	Non-flow system	136	8.3	0.285
					55 ^a	Non-flow system	136	8.5	0.290

Chemostat Number 10; Chemostat Volume 1,045 ml.

1	-	-	-	-	28	0.861	161	7.3	0.105
2	-	-	-	-	29	0.861	161	7.3	0.096
3	-	-	-	-	30	0.861	161	7.4	0.097
4	1.32	170	8.1	0.110	31 ^a	0.861	161	7.4	0.098
5	1.32	170	8.5	0.135	32	0.817	161	7.8	0.088
6	1.32	170	8.5	0.125	33	0.817	161	8.0	0.084
7	1.32	170	8.5	0.135	34 ^a	0.817	161	8.0	0.085
8 ^a	1.32	170	8.5	0.135	35	0.718	175	-	-
9	1.22	170	-	-	36	0.718	175	7.0	0.075
10	1.22	170	-	-	37	0.718	175	7.8	0.070
11	1.22	170	8.8	0.130	38	0.718	175	7.0	0.068
12	1.22	161	-	-	39	0.718	175	7.0	0.070
13	1.22	161	-	-	40	4.78	172	-	-
14	1.22	161	8.5	0.103	41	4.78	172	8.3	0.170
15	1.22	161	8.1	0.106	42	4.78	172	8.5	0.260
16 ^a	1.22	161	8.1	0.110	43	4.78	172	8.5	0.282
17	2.62	166	8.4	0.150	44	4.78	172	7.5	0.301
18	2.62	166	7.8	0.175	45 ^a	4.78	172	8.0	0.300
19	2.62	166	-	-	46	4.62	172	7.4	0.293
20	2.62	166	7.6	0.180	47 ^a	4.62	172	8.0	0.285
21 ^a	2.62	166	7.2	0.183	48	Non-flow system	172	-	-
22	2.61	166	7.5	0.157	49	Non-flow system	172	-	-
23	2.61	166	7.3	0.160	50	Non-flow system	172	-	-
24	2.61	166	7.2	0.165	51	Non-flow system	172	8.2	0.355
25	2.61	166	7.5	0.190	52	Non-flow system	172	8.1	0.360
26 ^a	2.61	166	8.1	0.190	53 ^a	Non-flow system	172	8.4	0.360
27	0.861	161	-	-	54	Non-flow system	172	8.3	0.365
					55 ^a	Non-flow system	172	8.5	0.365

^aSampling days.

CHEMOSTAT OPERATION DATA (Continued)

Chemostat Number 11; Chemostat Volume 1,055 ml

Time day	Residence Time days	S ₀ PO ₄ μg/l-P	pH Units	Absorbance OD/inch	Time day	Residence Time days	S ₀ PO ₄ μg/l-P	pH Units	Absorbance OD/inch
1	-	-	-	-	28	0.924	162	7.3	0.115
2	-	-	-	-	29	0.924	162	7.4	0.100
3	-	-	-	-	30	0.924	162	7.4	0.098
4	1.35	171	8.9	0.120	31 ^a	0.924	162	7.4	0.100
5	1.35	171	8.8	0.150	32	0.865	162	7.4	0.096
6	1.35	171	9.0	0.140	33	0.865	162	8.2	0.097
7	1.35	171	9.2	0.135	34 ^a	0.865	162	8.2	0.098
8 ^a	1.35	171	8.7	0.120	35	0.763	175	-	-
9	1.22	171	-	-	36	0.763	175	7.0	0.086
10	1.22	171	-	-	37	0.763	175	7.8	0.077
11	1.22	171	8.7	0.123	38	0.763	175	6.9	0.080
12	1.22	163	-	-	39	0.763	175	7.0	0.082
13	1.22	163	-	-	40	5.18	172	-	-
14	1.22	163	8.8	0.112	41	5.18	172	8.3	0.180
15	1.22	163	8.3	0.113	42	5.18	172	8.6	0.240
16 ^a	1.22	163	8.0	0.113	43	5.18	172	8.5	0.255
17	2.68	172	8.3	0.153	44	5.18	172	7.5	0.270
18	2.68	172	7.8	0.175	45 ^a	5.18	172	8.1	0.272
19	2.68	172	-	-	46	5.18	172	7.4	0.270
20	2.68	172	7.6	0.190	47 ^a	5.18	172	8.0	0.280
21 ^a	2.68	172	7.3	0.185	48	Non-flow	172	-	-
22	2.58	172	7.5	0.175	49	system	172	-	-
23	2.58	172	7.3	0.170	50	↓	172	-	-
24	2.58	172	7.2	0.173	51		172	8.0	0.335
25	2.58	172	7.5	0.195	52		172	7.8	0.330
26 ^a	2.58	172	8.0	0.190	53 ^a		172	8.0	0.335
27	0.924	162	-	-	54		172	8.3	0.330
					55		172	8.5	0.335

Chemostat Number 12; Chemostat Volume 1,055 ml

Time day	Residence Time days	S ₀ PO ₄ μg/l-P	pH Units	Absorbance OD/inch	Time day	Residence Time days	S ₀ PO ₄ μg/l-P	pH Units	Absorbance OD/inch
1	-	-	-	-	28	0.920	164	7.3	0.120
2	-	-	-	-	29	0.920	164	7.4	0.100
3	-	-	-	-	30	0.920	164	7.4	0.100
4	1.45	175	8.6	0.183	31 ^a	0.920	164	7.4	0.105
5	1.45	175	8.4	0.185	32	0.866	164	7.8	0.100
6	1.45	175	9.2	0.145	33	0.866	164	8.2	0.095
7	1.45	175	9.3	0.125	34 ^a	0.866	164	8.2	0.099
8 ^a	1.45	175	8.8	0.115	35	0.785	175	-	-
9	1.32	175	-	-	36	0.785	175	7.1	0.095
10	1.32	175	-	-	37	0.785	175	7.7	0.085
11	1.32	175	8.2	0.100	38	0.785	175	7.2	0.086
12	1.32	165	-	-	39	0.785	175	7.1	0.085
13	1.32	165	-	-	40	5.51	171	-	-
14	1.32	165	8.6	0.090	41	5.51	171	8.3	0.205
15	1.32	165	8.3	0.098	42	5.51	171	8.5	0.280
16 ^a	1.32	165	8.0	0.105	43	5.51	171	8.5	0.280
17	2.86	169	8.2	0.149	44	5.51	171	7.4	0.285
18	2.86	169	7.8	0.172	45 ^a	5.51	171	7.9	0.280
19	2.86	169	-	-	46	5.42	171	7.4	0.275
20	2.86	169	7.5	0.163	47 ^a	5.42	171	7.9	0.278
21 ^a	2.86	169	7.3	0.162	48	Non-flow	171	-	-
22	2.82	169	7.5	0.165	49	system	171	-	-
23	2.82	169	7.3	0.170	50	↓	171	-	-
24	2.82	169	7.2	0.172	51		171	7.8	0.330
25	2.82	169	7.5	0.185	52		171	7.7	0.330
26 ^a	2.82	169	8.0	0.175	53 ^a		171	8.4	0.340
27	0.920	164	-	-	54		171	8.4	0.345
					55		171	8.5	0.345

^aSampling days.